SHORT COMMUNICATION

More than we bargained for: Zebra mussels transported amongst European native freshwater snails

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Abstract

The international pet trade is a major driver of non-native species spread, including species both sold in the trade, and organisms incidentally transported alongside. Here, we document the discovery of invasive zebra mussels, *Dreissena polymorpha*, in Germany, transported alongside a commonly traded garden pond snail and European native, *Viviparus viviparus*, ordered from a German pet website. We highlight that the trade poses yet another way in which zebra mussels and other invasive species can expand their invaded range into novel ecosystems. We call for stricter biosecurity enforcement towards sellers, and encourage raising awareness amongst customers to inhibit the further spread of invasive species through the pet trade.

Keywords

Introduction

The international pet trade has facilitated the movement of organisms around the world, and is deemed responsible for a third of all aquatic non-native species (Padilla and Williams 2004), with escapes and releases from the aquarium trade a major pathway for non-native freshwater species in Europe (Nunes et al. 2015). Difficulties

surrounding regulation and enforcement (Patoka et al. 2018) are likely to increase as this global market continues to grow, with advances in technology increasing the availability of species from around the world, with websites and informal, peer-to-peer online marketplaces providing new purchasing options for customers (Olden et al. 2021). While the release and escape of traded species are often the focus of invasion ecologists (Kouba et al. 2021; Dickey et al. 2022), the risk of spreading "hitchhikers", i.e. fauna carried incidentally, has only recently received more interest (Duggan 2010). Indeed, recent studies have found the protozoan *Vorticella* sp. and a species of bdelloid rotifer associated with two species of atyid shrimps (Patoka et al. 2016), digenean larvae with the carnivorous gastropod *A. helena* (Stanicka et al. 2022), and an epibiont, *Diceratocephala boschmai*, on New Guinean ornamental *Cherax* crayfish (Lozek et al. 2021).

A high-profile example of an aquarium hitchhiker came in 2021, when zebra mussels (*Dreissena polymorpha*) were detected in 21 US states on aquarium moss balls that had been imported from Ukraine (United States Geological Survey Communications and Publishing 2021), and similar findings have emerged from Europe (Patoka and Patoková 2021). The zebra mussel is a Ponto-Caspian bivalve species that has colonized European and North American waters, and has been listed as one of the IUCN's 100 of the Worst Invasive Species (Lowe and Poorter 2000) due to its myriad economic (Connelly et al. 2007) and ecological impacts (Karatayev et al. 2002). By forming dense biogenic reefs, they compete with native unionids and zooplankton for planktonic food sources, and with fish for benthic space (Karatayev et al. 2002; Minchin et al. 2002). They also create hard-substrata in otherwise soft sediment environments, and affect water chemistry and clarity which in turn affects planktonic community, macrophyte coverage and food-web structure (Karatayev et al. 2002; Kirsch and Dzialowski 2012). With high byssal thread synthesis and attachment strength (Peyer et al. 2009), zebra mussels are capable of attaching to other organisms and boat hulls for overland transport and further spread (Collas et al. 2018). Here, we report the concerning arrival of zebra mussels amongst a delivery of a European native snail species, *Viviparus viviparus*, from an online pet store.

Methods and results

Discovery

Seventy-five *V. viviparus* (mean shell width ± standard error: 30.44 ± 0.39 mm; shell measured as per Fig. 2 in Jakubik and Lewandowski 2007) were ordered from a German online pet store (store name intentionally omitted). They arrived on the 8th April 2022, split across three plastic bags (n = 25 in each), in a polystyrene box. Upon arrival, they were taken to a climate control chamber (temperature 18 ± 1 °C), split into two 56 L glass aquaria holding tanks (60 cm × 30 cm × 30 cm length, width and height) containing 20 μm filtered freshwater and a filter, with thoroughly washed sand (1 cm deep) and white cockle shells for habitat. On the 9th April, two living freshwater mussels were found to be attached to two of the snails. These were subsequently measured
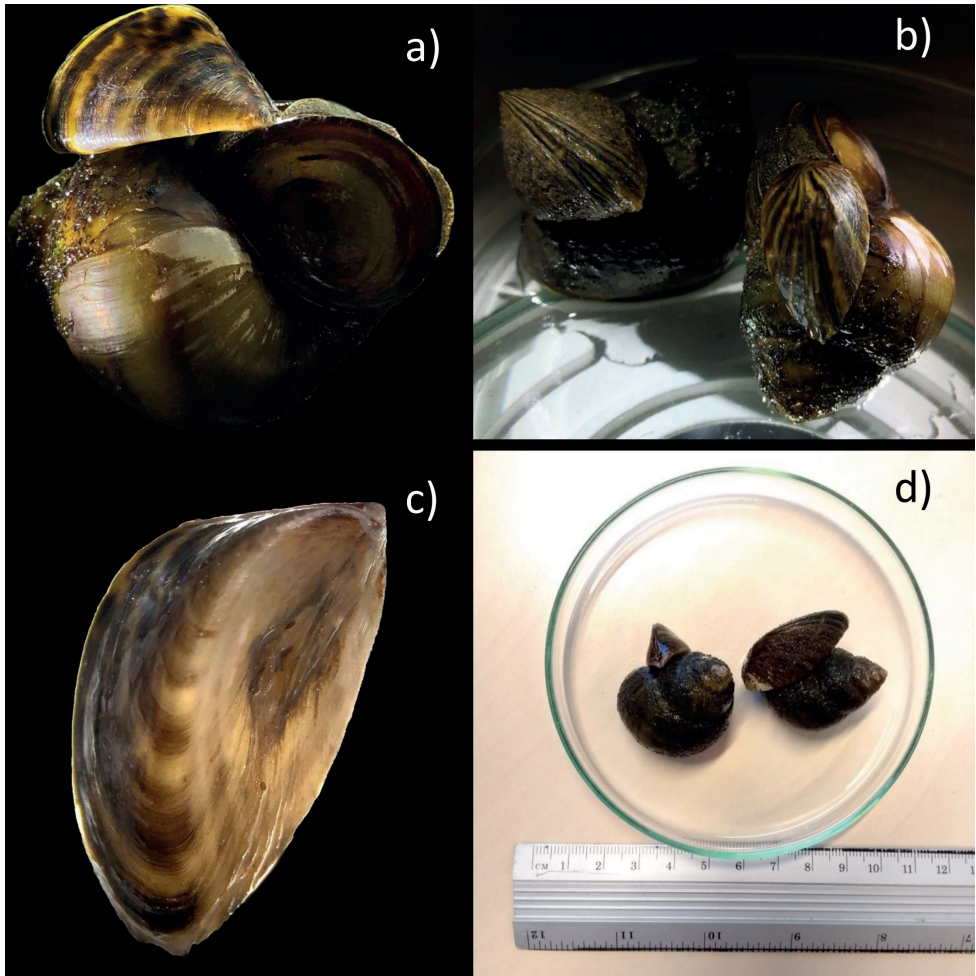
Figure 1. Zebra mussels, *Dreissena polymorpha*, found amongst ordered European pond snail, *Viviparus viviparus*.

with calipers (Mussel 1: length 30.7 mm, width 15.5 mm; Mussel 2: length 19.3 cm, width 11.0 mm – note both too large to have come from the laboratory water source), photographed (Fig. 1) and preserved in ethanol under refrigerated conditions for subsequent molecular identification.

DNA extraction, PCR and sequencing

DNA was extracted from the foot tissue of two mussel specimens using the DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. A fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) was amplified using primer pair LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994).
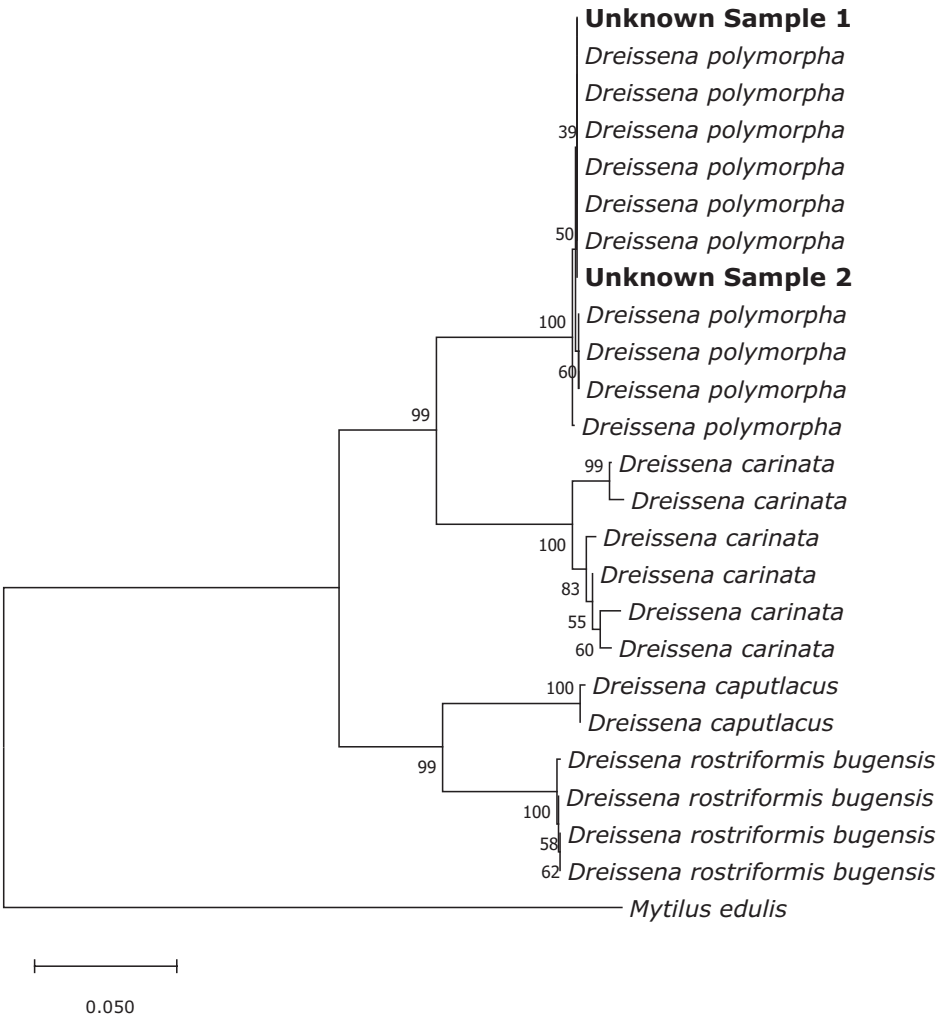
Figure 2. Neighbor joining tree from Mega. Bootstrap values are the percentage of trees supporting the shown topology with *Mytilus edulis* set as the root. The scale bar indicates the number of base differences per site. Unknown Sample 1 and 2 are the two mussels found attached to *Viviparus viviparus*. These results show that the unknown samples are *Dreissena polymorpha*.

PCR reactions were conducted in 10 μL volume reactions, containing 1 μL of forward and reverse primers (5mM concentration), template DNA, 10X PCR buffer (Invitrogen, USA) and dNTPs, 0.1 μL Taq DNA Polymerase (Invitrogen, USA) and 4.9 μL of nuclease-free water. Amplification was performed under the following conditions: 94 °C for 3 minutes; 35 cycles of 94 °C for 45 seconds, 48 °C for 45 seconds, and 72 °C for 60 seconds; 72 °C for 7 minutes. PCR products were sequenced on Sanger sequencing platform (Applied Biosystems, USA) at Eurofins Genomics (Kiel, Germany).

Sequencing results and analysis

Raw COI sequences were assembled and trimmed using CodonCode Aligner v 3.7.1 (Codon Code Corporation). Each sequence was blasted on NCBI (https://www.ncbi.nlm.nih.gov/) and BOLD (Ratnasingham and Hebert 2007). Sequences with ≥ 98% similarity were used as the preliminary identification results and implicated *D. polymorpha*. To verify the species identification, we constructed a phylogenetic tree by first downloading from BOLD ten sequences of *D. polymorpha*, two sequences of each additional *Dreissena* species found in BOLD (*D. carinata*, *D. rostriformis bugensis*, *D. caputlacus*), and one outgroup species (*Mytilus edulis*) (Suppl. material 1). All sequences were aligned using MUSCLE (Edgar 2004) in Unipro UGENE v37.0 (Okonechnikov et al. 2012). A phylogenetic tree was constructed using the neighbour-joining method and maximum likelihood in MEGA v10.1.8 (Kumar et al. 2018) with 10000 iterations, following default settings. The final analysis included 583 bases and 25 total sequences.

Alignments to databases of known samples (NCBI, BOLD) showed that the two mussels had high sequence similarity (> 98%) to *D. polymorpha*. Subsequent phylogenetic analysis further supported the assignment of these samples to *D. polymorpha*. Note that neighbour-joining and maximum likelihood (results not shown) revealed the same phylogenetic relationships. Therefore, we have high confidence that these samples are *D. polymorpha*.

Discussion

While concern surrounds the spread of commensal organisms, pathogens, and incidental organisms associated with non-native species in the pet trade (Patoka et al. 2020; Lozek et al. 2021; Stanicka et al. 2022), here we highlight the overlooked risk of native species in the trade facilitating the spread of non-native hitchhikers. *Viviparus viviparus* is a species distributed across Europe and advertised as being suitable for garden ponds. Escape from ponds is considered a major pathway for freshwater species introductions (Patoka et al. 2016), and accidental introductions of zebra mussels could lead to further dispersal through zoochorous means (Coughlan et al. 2017, 2019), or flooding events, as has been the case for aquaculture facilities in the past (Casimiro et al. 2018; McGlade et al. 2022). Indeed, questions surrounding the conditions under which *V. viviparus* were kept prior to shipping require answers, both to establish the biosecurity risk of that facility and to establish what other species in the pet and garden trade could be subject to similar hitchhiking. It may be that *V. viviparus* is unique as it is a European native capable of surviving the conditions under which it could come into contact with zebra mussels (i.e. it may have been held in outdoor ponds prior to collection and shipping), and in size, with a shell large enough for zebra mussels to attach to. However, zebra mussels have previously been found attached to the carapaces of crayfishes (Ďuriš et al. 2007), and the European native crayfish *Astacus astacus* is also sold for garden ponds in Germany and potentially held in outdoor stocking ponds

prior to shipment. With crayfish capable of overland dispersal and able to shed their mussel load upon moulting (Coughlan et al. 2017), the purchase of this species may pose an even greater risk of carrying zebra mussels and similar species (e.g. quagga mussels, *D. bugensis*).

Calls have been made for "white lists" of low-risk species that can be sold in the pet trade in place of risky species (Simberloff 2006; Patoka et al. 2018). However, we demonstrate that a native species (*V. viviparus*) can be a vector for ecologically detrimental invaders. Thus, even when a species itself is non-invasive and transported within its native range, and therefore immune to bans on trade stemming from legislation like the EU List of Union Concern, the potential for non-native hitchhikers can increase the ecological risk of nearly any traded organism. Indeed, Simberloff (2006) called for any white list species to be subject to "serious expert scrutiny" and we propose this should be the case for native species, with the ability to transport invasive species assessed within any potential risk assessments.

Greater biosecurity practices are also required, and need to be at the forefront of future policy revisions. The recommendations of Ložek et al. (2021), despite being focused on biosecurity measures surrounding the exportation of wild-caught individuals, could also prove effective in the case of species being held and/or bred in outdoor ponds. For example, the checking and disinfection of individuals collected from outdoor ponds, quarantining before transporting, and regular sanitation of outdoor stock ponds could help limit future incidental transport of invaders such as zebra mussels. Environmental DNA surveillance could be another solution for detecting invasive species at small abundances, as has been done effectively when assessing water samples from stores selling live bait for the DNA of invasive fish species (Nathan et al. 2015). Of course, species held in outdoor ponds could be host to other, unknown, less conspicuous hitchhikers, and the enforcement of intermittent stock health assessments could help limit the transportation of pathogens and parasites into novel ecosystems. Further, steps need to be taken to prevent the creation of more "dead letters", i.e. laws that exist but are not implemented (Patoka et al. 2018), and ensure enforcement. Beyond this, raising awareness amongst customers through a simple warning to check for unexpected organisms could provide an effective last line of defense.

Acknowledgements

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Supplementary material 1

List of species and accession numbers used for constructing phylogenetic trees

Authors: James W. E. Dickey, Reid S. Brennan, Sheena Suet-Wah Chung, Jonathan M. Jeschke, Gregor T. Steffen, Elizabeta Briski

Data type: table

Link: https://doi.org/10.3897/neobiota.83.97647.suppl1

Supplementary material 2

FASTA file of all sequences used to generate the phylogenetic tree

Authors: James W. E. Dickey, Reid S. Brennan, Sheena Suet-Wah Chung, Jonathan M. Jeschke, Gregor T. Steffen, Elizabeta Briski

Data type: FASTA file

Explanation note: The two unknown mussel samples (here confirmed to be *D. polymorpha*) have been uploaded to NCBI GenBank under accession numbers OP714457 and OP714458.

Link: https://doi.org/10.3897/neobiota.83.97647.suppl2

REVIEW ARTICLE

The Alien to Cyprus Entomofauna (ACE) database: a review of the current status of alien insects (Arthropoda, Insecta) including an updated species checklist, discussion on impacts and recommendations for informing management

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Abstract

Alien insects represent one of the most species rich groups of organisms introduced to Europe, with some responsible for adverse social-economic, human-health, biodiversity and ecosystem impacts. The impacts of invasive alien species, especially on island ecosystems, have been a hot topic of research worldwide. Cyprus is a Mediterranean island at the biogeographic crossroads of Asia, Africa and Europe. This study presents the database of the alien insects of the island of Cyprus as a whole, created through an extensive review including grey literature and online sources. The Alien to Cyprus Entomofauna (ACE) triples the known number of alien insects and adds supplemental information to existing species. Data concerning a total of 349 alien insects are presented alongside an updated checklist and recommendations for informing management. The status of alien insects on the island, their origin, trophic guilds, establishment, pathways of introduction and impacts are discussed. Developing an alien species inventory for the island is challenging

due to its geographic position and the increasing movement of people and goods leading to new species introductions. This publication constitutes an important first step towards providing information for effective actions to tackle invasive alien insects on Cyprus. The checklist and accompanying information can underpin understanding of the status and trends of alien species including providing information for risk assessments. ACE will continue to be maintained and updated as new records for Cyprus are made.

Introduction

The number of alien species across the world is increasing and showing no signs of saturation (Seebens et al. 2017, 2020; Seebens 2019). The present number of documented alien (non-native or exotic) species in Europe is approximately 14,000, one fifth of which are insects (EASIN 2021). A proportion of alien species are categorised as invasive because they threaten native biodiversity and ecosystem services and/or negatively affect human health, society and economy (Mazza and Tricarico 2018; Haubrock et al. 2021). According to the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), invasive alien species have been identified as one of the five main direct drivers of biodiversity change alongside land- and sea-use changes, exploitation of natural resources, climate change and pollution (IPBES 2019; Bellard et al. 2022). The economic cost of biological invasions in Europe, from 1960 to 2020, has been estimated to exceed €116.61 billion euros, despite the evident lack of data for many invasive alien species, urging for a comprehensive appraisal of costs (Haubrock et al. 2021).

The impact of biological invasions on island communities has received considerable attention (Reaser et al. 2007; Russell et al. 2017), with invasive alien species having severe adverse consequences on the evolutionary histories and extinction rates of island species (Mooney and Cleland 2001). Biogeographic research on island biological invasions has highlighted that there are higher numbers of alien species per area unit on islands compared to the mainland (Yamanaka et al. 2015; Dawson et al. 2017) and the number of alien species increases with degree of island isolation in contrast to the number of native species (Moser et al. 2018). It is predicted that there will be an increase in the introduction of alien species during the following decades, mainly driven by social-economic activities (e.g. trade and tourism) facilitating the arrival of stowaways and contaminants (Lenzner et al. 2020; Pergl et al. 2020).

The island of Cyprus is situated at the eastern Mediterranean Sea and bordered by three continents. Its socio-political background has resulted in the classification of the island sometimes as a part of Europe (being part of the European Union) and its geographical position as a Middle Eastern or Western Asian country. The first human-mediated introduction of organisms to the island dates back to 10,500–9000 BC, when the first settlers introduced to Cyprus economically important fauna (i.e. livestock and

game animals) as well as horticultural flora (Zeder 2008). The proximity of the island to three continents, the continuous trade over millennia and the global increase in the import of goods and movements of people (Hulme 2009; Demesticha 2019; Seebens 2019), provide opportunities for alien species to arrive in Cyprus (Seebens et al. 2018) and contribute to the challenges for Cyprus in tackling biological invasions.

The alien insect fauna of the island was first documented through DAISIE (2009) and Roques et al. (2010), which reported 114 "confirmed alien" and "cryptogenic" species. In 2020, this number was supplemented by Martinou et al. (2020) reaching a total of 123 species, through the development of the Cyprus Database of Alien Species (CyDAS – www.ris-ky.info/cydas). However, this number was considered to be a significant underestimate, given the species richness of insects. Thus, the need for the compilation and construction of an up-to-date database integrating data from various sources was identified. The Alien to Cyprus Entomofauna (ACE) database provides information on alien insects of the island of Cyprus, subsequently contributing data to the CyDAS.

Materials and methods

Species checklists and databases compiled by DAISIE (2009), Roques et al. (2010), Martinou et al. (2020) and EASIN (2021) were used as a foundation for the database which was extended through a literature survey for records of alien insects in Cyprus. Records of alien species in Cyprus were searched through Google Scholar using the keywords "species name" and "Cyprus". Data were extracted from peer-reviewed journal articles [e.g. Wood (1963); Háva et al. (2010); Collins and Philippou (2016); Salata et al. (2019); Davranoglou et al. (2020, 2021)], reviews [e.g. Greathead (1976)], institutional reports [e.g. FAO (1996); EPPO (1997)], books and book chapters [e.g. Georghiou (1977); Gerber and Schaffner (2016); Sparrow and John (2016)], as well as online sources [e.g. Srour (2013); Fägerström (2021)]. Literature surveys were completed on 31 December 2021.

The status of species was assessed as either "confirmed alien species" or "cryptogenic", with the latter term referring to taxa of unknown origin, neither demonstrably native nor introduced (Carlton 1996). A third category labelled "questionable" was used following EASIN (2021), addressing species whose status should be further investigated. Ten species were added to this category, including species regarded as native to Northern Africa, the Middle East or Western Asia which have not been knowingly introduced to the island such as the beetle *Coccotrypes dactyliperda* (Fabricius, 1801) (Georghiou 1977; Spennemann 2019), the hemipteran *Jacobiasca lybica* (Bergevin & Zanon, 1922) (Georghiou 1977), the dipteran *Pseudodoros nigricollis* Becker, 1903 (van Eck and Makris 2016; André van Eck pers. comm.), the lepidopteran *Dichelia cedricola* (Diakonoff, 1974) (Gatzogiannis et al. 2010) and the hymenopterans *Xylocopa* (*Koptortosoma*) *pubescens* Spinola, 1838 (Varnava et al. 2020), *Aphytis coheni* DeBach, 1960 (Wood 1963), *Diversinervus elegans* Silvestri, 1915 (Orphanides 1988), *Microterys nietneri* (Motschulsky, 1859) (Wood 1963), *Scutellista caerulea* (Fonscolombe, 1832)

(Georghiou 1977; Gerber and Schaffner 2016) and *Vespula germanica* (Fabricius, 1793) (Morris 1937).

Occasional migrants (mainly Lepidoptera and Orthoptera), i.e. lepidopterans *Spoladea recurvalis* (Fabricius, 1775) (De Prins 2005; Lopez-Vaamonde et al. 2010), *Catopsilia florella* (Fabricius, 1775) (John et al. 2019) and *Danaus chrysippus* (Linnaeus, 1758) (Georghiou 1977; Lopez-Vaamonde et al. 2010), as well as orthopterans *Locusta migratoria* (Linnaeus, 1758) and *Schistocerca gregaria* Forsskål, 1775 (Rasplus and Roques 2010; Siedle et al. 2016), were excluded. In addition, species treated by various databases as alien to Europe (Roques et al. 2010; EASIN 2021), but were found to be native to Cyprus, were also excluded. These species were *Acheta domesticus* (Linnaeus, 1758) native to south-western Asia (Rasplus and Roques 2010; Siedle et al. 2016), the northern African aphid *Cinara cedri* Mimeur, 1936 (Coeur d'Acier et al. 2010) represented by the endemic subspecies *Cinara cedri brevifoliae* A.Binazzi, 2017 (Binazzi et al. 2017) and two Asian chalcid wasps, *Aphidius colemani* Viereck, 1912 and *Megastigmus schimitscheki* Novitzky, 1954 (Rasplus et al. 2010; Auger-Rozenberg et al. 2012; Gerber and Schaffner 2016).

The native range of a species was assigned according to biogeographic realms (Udvardy 1975; Snow and Perrins 1998), including species native to tropical and subtropical regions in a category derived verbatim from Roques (2010). In cases of species native to multiple biogeographic realms, all biogeographic realms were documented. Species of "cryptogenic" and "questionable" status were excluded. This treatment was recently applied in a similar publication for the alien insects of Greece (Demetriou et al. 2021). Species were assigned to broad trophic guilds covering phytophagous, detritivorous, parasitic and predatory insects (Roques et al. 2010). Phytophagous insects were subsequently categorised in the following classes considering their main feeding patterns: pollinators, leaf miners, gallers (including leaf- and seed gall-inducers), insects with chewing mouthparts (feeding on flowers, stems, leaves and soft tissues), insects with sucking mouthparts (taxa with sucking mouthparts, feeding on sap) and wood feeders (wood borers and xylem eating insects).

The establishment status was assessed as follows: "Established" (sustaining populations on the island); "Failed to establish" (unintentionally introduced, but failed to establish); "Released, but failed to establish" (intentionally released, but failed to establish); "Eradicated" (confirmed eradication); "Doubtful" (species potentially wrongly identified or records regarded dubious); and "Unknown" (establishment status could not be assigned because of lack of data or species or reported only once). Establishment status was assessed through literature surveys as well as species occurrences by citizen-scientists in the iNaturalist collection project "Alien to Cyprus Entomofauna" (https://www.inaturalist.org/projects/alien-to-cyprus-entomofauna) (iNaturalist 2022), the Global Biodiversity Information Facility (GBIF 2022) and the authors.

Intentional introductions (releases) of biological control agents to the island were catalogued, reporting on their taxonomy, import year, establishment status and recorded impacts on target and non-target species. A preliminary assessment of recorded and inferred impacts of alien insects in Cyprus, was undertaken according to the categories devised by Kenis and Branco (2010). Specifically, we analysed environmental

and socio-economic impacts, with the latter category being further divided into alien insects identified as "Outdoor agricultural and horticultural pests", "Pests of protected horticulture", "Stored product and infrastructure pests", "Forestry and urban tree pests", "Arthropods affecting human and animal health", as well as "Arthropods with a positive economic impact". In cases where no impacts were recorded or data were insufficient for a robust classification, species were treated as data deficient. Species which displayed both positive and negative impacts or could be assigned into more than one subcategories of negative socio-economic impact, were assigned accordingly in all applicable impact subcategories.

Results

Biodiversity and species richness

According to the literature search, a total of 349 alien species were identified (Suppl. material 1), distributed within 261 genera and 97 insect families (Fig. 1). The current number of alien insects of Cyprus has tripled (204% increase) since their first treatment (DAISIE 2009; Roques et al. 2010; Martinou et al. 2020). Out of these species, only one can be found in the EPPO A1 list of pests recommended for regulation as quarantine pests, namely *Spodoptera litura* (Fabricius, 1775), while 12 can be found in the EPPO A2 list (Suppl. material 1).

Status

Of the total number of species, most of them are "confirmed alien species" to the island (242 species = 69%), while more than one fourth (97 species = 28%) are "cryptogenic" and ten species (3%) were classified as "questionable" (Fig. 2).

Origin

The largest percentage of "confirmed alien" insect species originates from the Indomalayan biogeographic realm (29%), followed by the Eastern Palearctic (15%). Each of the Afrotropical and Australian realms contribute 14% of "confirmed alien species". Nearly one fifth of "confirmed alien species" originate from the New World, being native to the Neotropical (12%) and Nearctic (10%) realms. Species originating from the tropics and subtropics (4%), as well as "confirmed alien species" within the Western Palearctic (3%), had the lowest representation within the dataset (Table 1).

Trophic guilds

Almost half of the alien insects in Cyprus are classified as phytophagous (48%). Almost one in four are detritivores (24%), while the remaining quarter accounts for parasites, parasitoids (grouped) (17%) and predators (11%) (Fig. 3). Only one species, the ant-
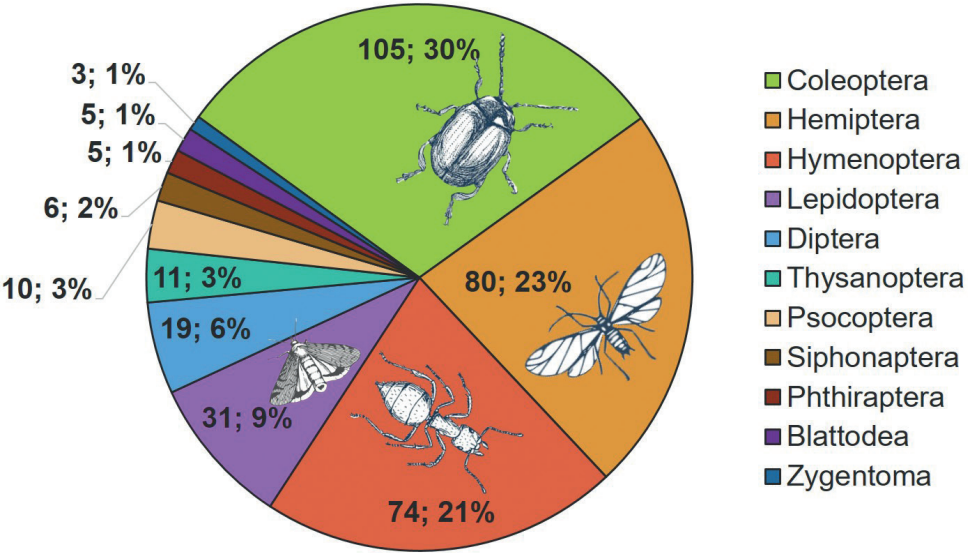
Figure 1. Number and percentage of alien insect species by order detected in Cyprus.
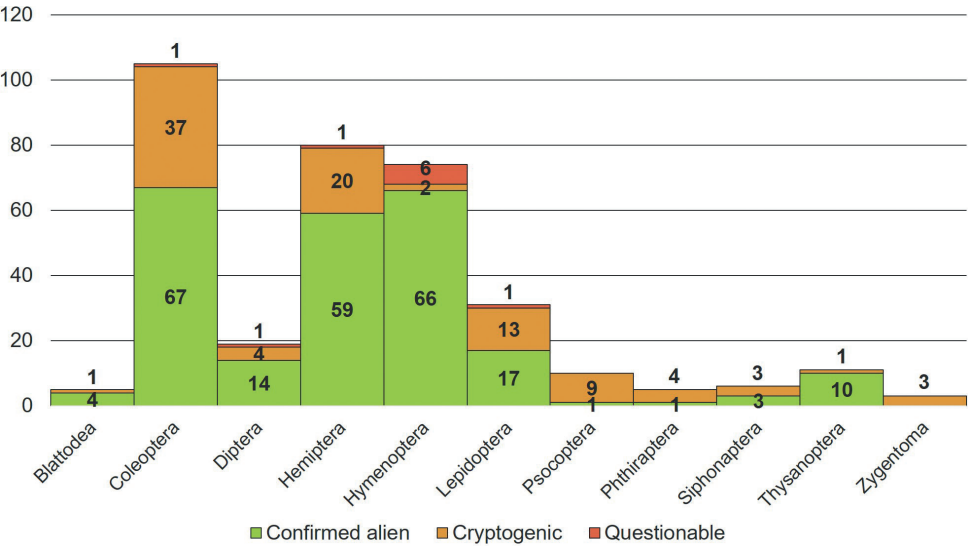
Figure 2. Status of alien insects species by order detected in Cyprus, classified as "confirmed alien" (truly demonstrated to be non-native to Europe and Cyprus), "cryptogenic" (species of unknown origin) and "questionable" (species whose status should be further investigated).

like beetle *Anthicus crinitus* La Ferté-Sénectère, 1849 was recorded as of unknown feeding habits (Denux and Zagatti 2010) and was subsequently excluded from the analysis.

Phytophagous alien insects were further classified into six functional groups (Table 2). Half of the represented phytophagous insects have sucking mouthparts,

Table 1. Origin [regions classified following Udvardy (1975) and Snow et al. (1998)] of orders of alien insects of Cyprus, excluding "cryptogenic" and "questionable" species.

|	Western Palearctic	Eastern Palearctic	Afrotropical	Indomalayan	Australian	Nearctic	Neotropical	Tropical / Subtropical
Blattodea	0	1	2	1	0	0	0	0
Coleoptera	3	12	10	22	9	4	9	6
Diptera	0	1	6	0	1	5	1	0
Hemiptera	1	15	6	17	5	9	11	4
Hymenoptera	3	11	12	29	20	6	9	0
Lepidoptera	0	3	2	8	4	1	3	0
Psocoptera	0	0	0	0	0	0	0	1
Phthiraptera	0	0	0	0	0	0	1	0
Siphonaptera	1	0	1	0	0	0	0	1
Thysanoptera	0	0	1	3	1	3	1	1
Zygentoma	0	0	0	0	0	0	0	0
Total	8	43	40	80	40	28	35	13

Table 2. Number of phytophagous alien species (within insect orders) within different functional groups.

|	Chewers	Gallers	Leaf miners	Pollinators	Suckers	Wood feeders
Coleoptera	31	0	0	0	0	10
Diptera	6	0	4	0	0	0
Hemiptera	0	0	0	0	78	0
Hymenoptera	1	9	0	5	0	0
Lepidoptera	16	0	4	0	0	1
Thysanoptera	0	0	0	0	7	0
Total	54	9	8	5	85	11
Total (%)	31	5	5	3	50	6

predominantly Hemiptera (92%) and some Thysanoptera (8%). Almost one third chew on leaves, stems and other soft tissues, mostly Coleoptera (57%) and Lepidoptera (30%). All leaf-, seed-gallers and pollinators are hymenopterans, whereas leaf-miners are equally divided between Diptera (Cecidomyidae) and Lepidoptera (Gelechiidae and Gracillariidae). Lastly, the majority of wood-feeding insects were from the order Coleoptera with just one moth from the family Castniidae (Table 2, Fig. 3).

Establishment status

Overall, most of alien insects (70%) seem to have established (producing viable, self-reproducing populations) on the island. Only two species, *Octodonta nipae* (Maulik, 1921) and the yellow fever mosquito *Aedes aegypti* Linnaeus, 1762 are considered to have been eradicated (1%). The Groundnut bruchid *Caryedon serratus* (Olivier, 1790) and the mango seed weevil *Sternochetus mangiferae* (Fabricius, 1775) were unintentionally introduced, but failed to establish (1%). The red scale parasitic wasp *Aphytis holoxanthus* DeBach, 1960 was intentionally released, but failed to establish and nine
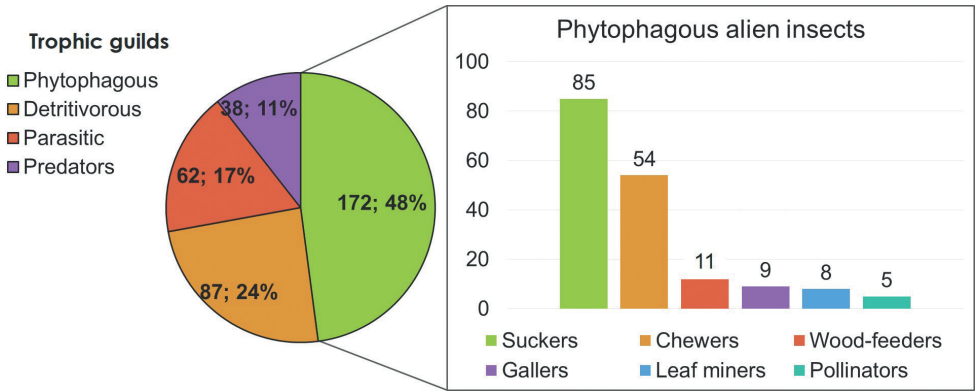
Figure 3. Trophic guilds of alien insects of Cyprus. The number of species and their percentages are shown on the pie chart. Further information on phytophagous insects and their classification is provided in the box depicting the overall number of species in each ecofunctional group.
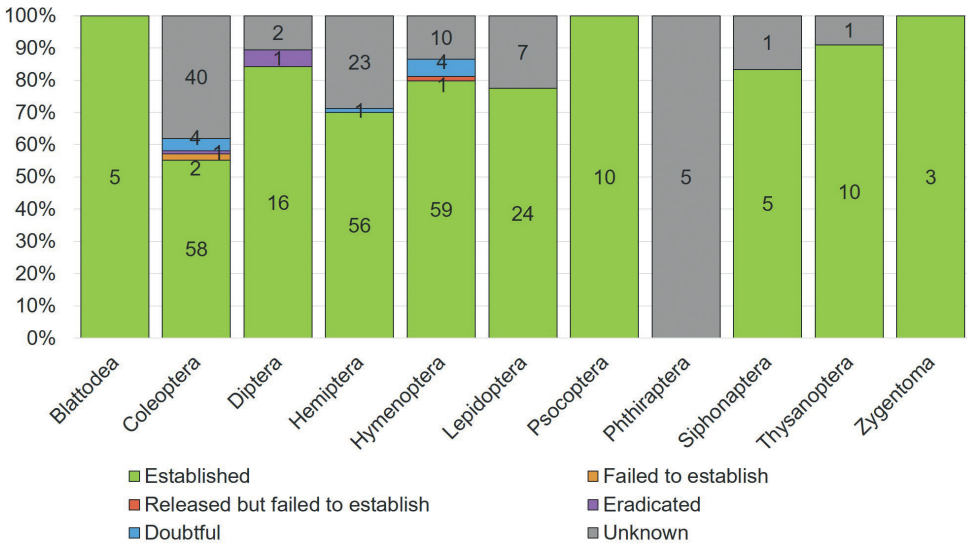
Figure 4. Number and percentage of alien insect species by order detected in Cyprus according to their establishment status, classified as "established" (sustaining populations on the island); "failed to establish" (unintentionally-introduced, but failed to establish); "released, but failed to establish" (intentionally released, but failed to establish); "eradicated" (confirmed eradication); "Doubtful" (species potentially wrongly identified or records regarded dubious); and "Unknown" (establishment status could not be assigned because of lack of data or species or reported only once).

species (3%) are considered doubtful or dubious. More information regarding their establishment status is given below. Nevertheless, information about the establishment of more than one quarter of alien insects (25%) remains unknown (Fig. 4).

## Intentional introductions – Biological control agents

Collectively, 32 alien biological control agents of crop pests have been intentionally introduced to Cyprus, accounting for approximately 9% of all alien insects, comprising five alien Coleoptera (16%) and 27 Hymenoptera (84%). Within the Hymenoptera, 17 species (63%) belong to the superfamily Chalcidoidea, nine (33%) to Ichneumonoidea and one species (4%) to the family Vespidae (Suppl. material 2).

Of the total species list, 26 species (81%) have established populations on the island. *Aphytis holoxanthus*, a parasitoid released for the control of scale insects, failed to establish soon after its import and release (Greathead 1976). The establishment status of four species (13%) is unknown. The presence of *Cirrospilus ingenuus* Gahan, 1932, parasitoid of citrus leaf miners, is considered doubtful being catalogued both as established, as well as not established (Gerber and Schaffner 2016).

Most of the introduced biological control agents have been released for control of pests in citrus (59%), potato (30%) and olive (9%) pests (Fig. 5). A single record suggesting the introduction of the yellow jacket *Vespula germanica* to the island (Morris 1937) needs confirmation. Half of these biological control agents were imported to the island during the decade 1951–1960, against potato and citrus pests (Fig. 5). From the
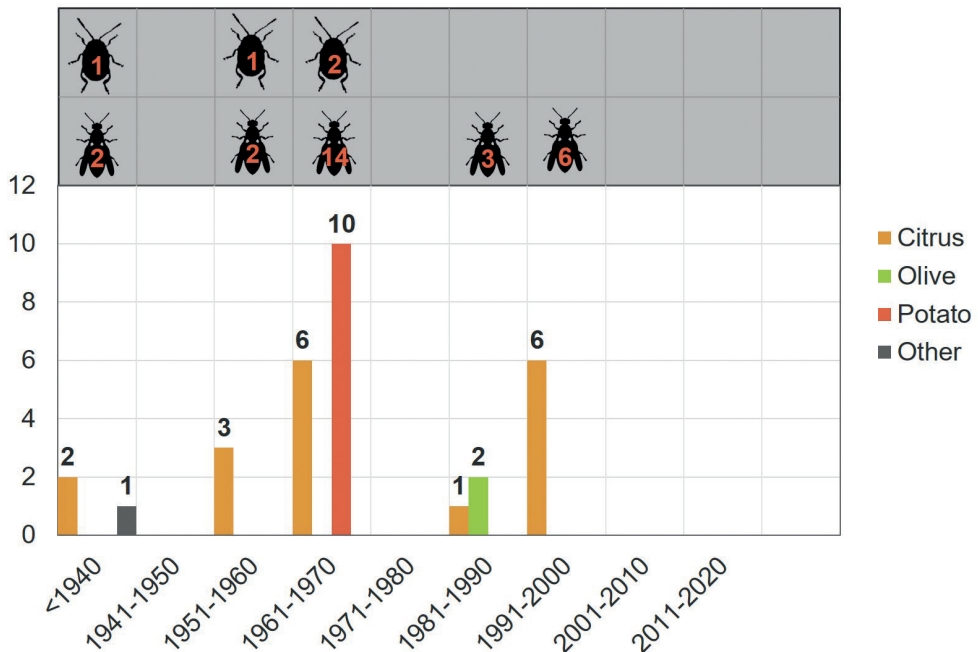
Figure 5. Introduction history of imported biological control agents to Cyprus. The graph shows the number of species per decade released to control citrus, olive, potato and other pests. The number of Coleoptera and Hymenoptera species introduced during each decade are shown in the box over the graph.
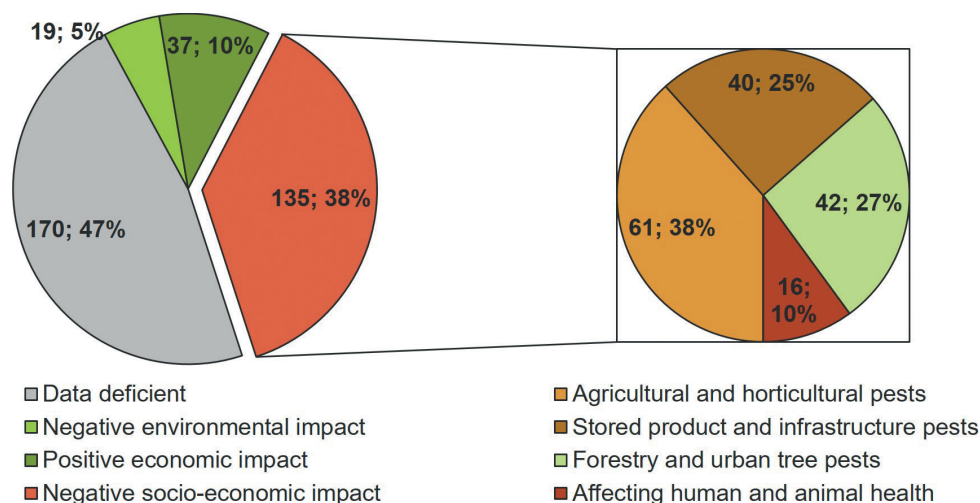
Figure 6. Known registered impacts of alien insects in Cyprus. The number of species classified as data deficient, having positive economic or negative environmental or socio-economic impacts, as well as their percentage are shown in the left pie chart. Negative socio-economic impacts are further divided into alien insects identified as "Outdoor agricultural and horticultural pests", "Pests of protected horticulture", "Stored product and infrastructure pests", "Forestry and urban tree pests" and "Arthropods affecting human and animal health" (right pie chart). The total number of species in this figure does not total to 349 as species displaying both positive and negative impacts or that could be assigned to more than one subcategories of negative socio-economic impacts.

searches conducted to date, it appears that there have been no official records of biological control agents intentionally released into the wild since the beginning of the 21st century. The introduction year of *Rhyzobius forestieri* (Mulsant, 1853), Forestier's ladybird, is unknown. Although Gerber and Schaffner (2016) cite an annual report of the Cyprus Agricultural Research Institute published in 1984, Orphanides (1988) does not mention the species in his article as a biological control agent of *Saissetia oleae* (Olivier, 1791) in Cyprus. Thus, the presence of *R. forestieri* on the island is considered dubious.

Impacts

Only 19 species (5% of total alien insects in Cyprus) were identified as invasive alien species, having negative impacts upon biodiversity and ecosystem functions (Fig. 6). Thirty-seven species (10%) had a positive socio-economic impact, negatively affecting invasive alien host-animal or -plant species, being pollinators or contributing towards the biological control of injurious or alien pest species. The majority of species with recorded negative impacts affected socio-economic parameters (135 species – 38%). These species were predominantly "agricultural and horticultural pests" (61 species – 38%), "forestry and urban tree pests" (42 species – 27%), "stored product and infrastructure pests" (40 species – 25%) and "insects affecting human and animal health" (16 species – 10%) (Fig. 6).

Most of the alien insects of Cyprus (170 species – 47%) were catalogued as data deficient due to the lack of studies addressing their impacts, the lack of observed impacts or the low quality of evidence for impacts (Fig. 6).

Discussion

Biodiversity and species richness

Coleoptera represent the most species-rich order of alien insects on Cyprus comprising more than 100 alien species (Fig. 1). Five families of Coleoptera, namely Nitidulidae (10%), Dermestidae (10%), Chrysomelidae (10%), Ptinidae (10%) and Curculionidae (9%), contain almost half of the alien beetles found in the island. These families include solely detritivores and phytophagous species found in stored products, such as the carpet beetles *Trogoderma granarium* Everts, 1898 and *Trogoderma versicolor* (Creutzer, 1799), the seed beetles *Bruchus rufimanus* Bohemann, 1833 and *Callosobruchus chinensis* (Linnaeus, 1758), as well as representatives of the genus *Sitophilus* Schoenherr, 1838 (Morris 1937; Georghiou 1977). Interestingly, the only references to *Sitophilus sculpturatus* (Gyllenhal, 1838) in Europe concern Cyprus nearly a century ago, when the species was reared from *Eugenia jambolana* (L.) Skeels seeds imported from South Africa (Morris 1937; Georghiou 1977). Nitidulids have been identified both as herbivores and detritivores feeding on ripe and rotten fruit (Georghiou 1977; Jelínek et al. 2016). In addition, these families include some easily detectable major pests of ornamental plants, such as the destructive red palm weevil *Rhynchophorus ferrugineus* (Olivier, 1790) (Kontodimas et al. 2006), but also the leaf beetle *Chrysolina americana* (Linnaeus, 1758), found damaging five aromatic Lamiaceae, including three species native to the island (Hadjiconstantis and Zoumides 2021). Despite the small body size of most species in the aforementioned families, their predominance in the list of alien Coleoptera may well be attributed to their peridomestic lifestyle and negative economic impacts on stored products, crops and ornamentals which reinforce the need for studies addressing their identification and approaches for mitigation of their negative impacts.

As is the case with Greece (Demetriou et al. 2021), Hemiptera are predominantly represented by scale insects (Coccoidea) (44%), followed by aphids (28%) and whiteflies (10%). The high numbers of alien Sternorrhyncha are strongly correlated to their unintentional transport as contaminants on infested plant material (Rabitsch 2010a), but also biological traits facilitating successful biological invasions, such as their minuscule body size, their ability to reproduce both through parthenogenesis and sexual reproduction, as well as their high fecundity (Coeur d'Acier et al. 2010; Pellizzari and Germain 2010). Furthermore, their host plants range includes a wide variety of economically important species increasing detection probabilities (Coeur d'Acier et al. 2010), although their ability to exploit "hidden" microhabitats (e.g. undersides of leaves, shoot and bark crevices) hinder their interception during phytosanitary inspections (Pellizzari and Germain 2010). Improving phytosanitary measures and

quarantine inspections, while recognising the challenges, may minimise the import of alien Hemiptera to the island as most species have been associated with common alien ornamental and agricultural plants (Georghiou 1977; Şişman and Ülgentürk 2010; Ülgentürk et al. 2015).

The superfamily Chalcidoidea, holds 71% of all recorded alien hymenopterans. The superfamilies Ichneumonoidea and Formicoidea follow, accounting for 14% and 12% of species, respectively. More than one third of alien Hymenoptera have been intentionally introduced to the island. Introduction pathways of the remaining Hymenoptera are currently unknown and most probably reflect unintentional introduction alongside their hemipteran hosts [e.g. *Psyllaephagus bliteus* Riek, 1962, an Australian parasitoid of the red gum lerp psyllid *Glycaspis brimblecombei* Moore, 1964; (Karaca et al. 2017)] or host plants [e.g. *Pleistodontes imperialis* Saunders, 1882, a mutualistic pollinator of Australian *Ficus rubiginosa* Desf. & Vent.; (Compton et al. 2020a)]. Nine species of alien ants have been collected from Cyprus, including the dubious records (possible misidentifications) of *Cardiocondyla nuda* (Mayr, 1866) (Bernard 1956), *Trichomyrmex destructor* (Jerdon, 1851) and the fire ant *Solenopsis geminata* (Fabricius, 1804) (Georghiou 1977; Collingwood et al. 1997; Salata et al. 2019). According to Salata et al. (2019), the Pharaoh ant *Monomorium pharaonis* (Linnaeus, 1758), *Nylanderia jaegerskioeldi* (Mayr, 1904) and the fire ant *S. geminata* are responsible for the elimination of native species within invaded habitats due to the aggressive behaviour of the invasive ants. Although the fire ant *S. geminata* is only known from an old, possibly erroneous literature record, Cyprus is stated to be within the species' known distribution (Collingwood et al. 1997; Dr Christos Georgiadis, pers. comm.).

All but one of the alien Lepidoptera are moths, with half of species falling under Pyralidae (19%), Gelechiidae (16%) and Tineidae (16%). These families include minute species commonly identified as stored product and household pests, such as the snout moths *Corcyra cephalonica* (Stainton, 1866) and *Ephestia elutella* (Hübner, 1796) and gelechiid moths *Pectinophora gossypiella* (Saunders, 1844) and *Sitotroga cerealella* (Olivier, 1789) (Morris 1937; Georghiou 1977). The sole exception is *Papilio demoleus* Linnaeus, 1758, a large alien butterfly reported only recently from Cyprus, but its impact on native biodiversity is still unknown (John et al. 2021, 2022).

Status

Due to the geographic location of Cyprus, surrounded by Europe, Africa and Asia and the lack of literature data, the status of ten species was treated as "questionable" (Fig. 2). The soldier fly *Pseudodoros nigricollis* is believed to be native to the East Mediterranean and Afrotropics (van Eck and Makris 2016; André van Eck, pers. comm.). The recent discovery of the species on the island and the general lack of knowledge surrounding the distribution of Syrphidae in the Near East pose difficulties in assessing the native or "alien" status of *P. nigricollis* in Cyprus (André van Eck, pers. comm.). In addition, the "cryptogenic" status of its associate host *Hyalopterus pruni* (Geoffroy, 1762) and its observed relationship with both native (*Phragmites australis* and *Prunus* spp.) and

alien host plants (*Musa* sp.), further complicate this assessment (André van Eck, pers. comm.). The date stone beetle, *Coccotrypes dactyliperda*, has been regarded as alien to Europe originating from some undetermined tropical or subtropical area (Sauvard et al. 2010). A more recent study characterises this species as of Middle Eastern origin (Spennemann 2019), but its main host plant *Phoenix dactylifera* L. is regarded as introduced to Cyprus (Christofides 2017). The planthopper *Jacobiasca lybica* probably originates from northern Africa, but is widely distributed in the Mediterranean (Mifsud et al. 2010). In Cyprus, the first and only record for the species dates back to 1967 when *J. lybica* was collected on grapes (Georghiou 1977). Previous studies failed to locate the species in the island (Lindberg 1948) and its "alien" status has been regarded as doubtful (Mifsud et al. 2010). The Asian moth *Dichelia cedricola*, has been labelled as alien to Europe (Lopez-Vaamonde et al. 2010). The species is a renowned pest of *Cedrus* spp. causing serious defoliation of *Cedrus libani* A. Rich. in neighbouring Lebanon and Turkey (Nemer et al. 2015). In Cyprus, management of the species in Cedar Valley, where the endemic cedar *C. brevifolia* occurs, was initiated a decade ago (Gatzogiannis et al. 2010). The isolation of Cedar Valley, situated deep within the islands' Troodos mountain range, the reduced available habitat for *D. cedricola* and nativity of similarly perceived alien associate of cedar *C. cedri*, may indicate that, not only *D. cedricola* could be native to Cyprus, but also consist an endemic subspecies.

In relation to Hymenoptera of "questionable" status, six species are presented. The Aphelinidae *Aphytis coheni* has been reported as of both western and south-eastern Asian origin (Avidov et al. 1970; Gerber and Schaffner 2016; EASIN 2021). Despite being intentionally introduced to the island as a biological control agent for *Lepidosaphes beckii* (Newman, 1869), the type-locality of *A. coheni* in neighboring Israel raises doubts about its region of ancestry (Gerber and Schaffner 2016). In Europe, *Scutellista caerulea* was released in France and Greece against *Saissetia oleae* (Olivier, 1791) (Gerber and Schaffner 2016). The species has been collected from Cyprus since 1931 (Wood 1963; Georghiou 1977), but there is no evidence of intentional introduction to the island. A native population of this African species already existed in Crete prior to the species' intentional introduction (Gerber and Schaffner 2016). Taking into account the proximity of both Crete and Cyprus to northern Africa, *S. caerulea* may indeed be native to Cyprus. The same principle applies to both African Encyrtidae *Diversinervus elegans* and *Microterys nietneri* released in Europe as biological control agents, but collected in Cyprus without a recorded history of intentional introductions (Wood 1963; Orphanides 1988; Gerber and Schaffner 2016). Regarding *Vespula germanica*, as stated in Morris (1937) "is said to have been introduced to the island some years ago in hopes of reducing the number of flies". To date, this statement remains unconfirmed. Despite being widespread and common in the Western Palearctic, *V. germanica* has been introduced to various islands, such as Iceland, Madeira and Canary Islands (Rasplus et al. 2010; Beggs et al. 2011). It is, therefore, not possible to confirm the native or "alien" status of *V. germanica* in Cyprus. Nevertheless, this hypothesis could be tested through molecular population genetics. Finally, *Xylocopa pubescens* is perhaps the most common carpenter bee species observed in Cyprus. Despite extensive research on the island's bee

fauna conducted during the 1940s and 50s by Georgios Mavromoustakis, the species was not recorded (Mavromoustakis 1949[1948], 1951, 1952). Earliest records of this large and easily identifiable species in Cyprus emerged after the 1990s (Terzo and Rasmont 2014; Varnava et al. 2020). There are no known cases of intentional introduction of *X. pubescens* or other bees on the island (Cyprus Veterinary Services – Ministry of Agriculture, Rural Development and the Environment, pers. comm.). The import of Apoidea from EU countries must be registered in the TRACES platform (https://food.ec.europa.eu/animals/traces_en), while introductions from third countries are mediated by health certificates and inspections upon arrival from custom controls. The Ethiopian ancestral origin of *X. pubescens*, recent detection of the species in 2012 from Athens, Greece (Terzo and Rasmont 2014) and its positive role as a pollinator of greenhouse crops in Israel (Sadeh et al. 2007), may reflect a recent range expansion or perhaps its introduction to the island for agricultural purposes.

Origin

Species originating from biogeographic realms surrounding the island, i.e. the Eastern and Western Palearctic, the Afrotropics and tropical/subtropical regions, account for more than one third (36%) of the "confirmed alien" insects of Cyprus. However, introductions from remote regions seem to have a strong influence on the composition of the island's "confirmed alien" entomofauna, with Indomalaya accounting for more than one fourth (29%) of "confirmed alien species" of insects (Table 1). Asian species, originating from Eastern Palearctic and Indomalaya represent the majority of introduced taxa (44%). This may derive from increased imports from Asian countries, such as China, Israel and Turkey, although the island's largest trading partners correspond to European countries, predominantly Greece and Italy (Trend Economy 2021). Therefore, Greece and Italy may contribute to the introduction of alien species to Cyprus, hidden as stowaways in shipment or avian cargo (Inghilesi et al. 2013; Avtzis et al. 2017; Demetriou et al. 2021).

As trade plays a crucial role in the introduction of alien species (Hulme 2009; Seebens 2019), enhancing biosecurity for regions with high import rates could be advantageous. This could include ongoing effective inspection mechanisms deployed through customs and border controls, at entry points such as airports and harbours, alongside implementation of specialised inspection protocols according to cargo type and origin.

Trophic guilds

The diversity of functional groups represented within each insect order reveals the range of alien insects of Cyprus. Detritivorous species are predominantly beetles (62%). To a lesser extent are Lepidoptera and Psocodea (12% each), followed by Diptera (6%) and common house intruders in the orders Blattodea and Zygentoma (6% and 3%, respectively). The overwhelming majority of parasitic taxa are Hymenoptera (79%), mostly

wasps combating agricultural pests of economic significance, such as the Neotropical braconid *Apanteles subandinus* Blanchard, 1947 tackling the common and destructive moth *Phthorimaea operculella* (Zeller, 1873) (Georghiou 1977; Gerber and Schaffner 2016) and others discussed below. These are followed by just a few animal parasites in the orders Siphonaptera and Phthiraptera, as well as some parasitic flies, such as *Trichopoda pictipennis* Bigot, 1876 (Kazilas et al. 2020; Dios et al. 2021). Most of the predatory species belong to Coleoptera (50%) and ants (26%). Predatory behaviour of alien insects has received little to no interest in Cyprus, except from alien Coccinellidae (Wood 1963). Five out of six alien ladybirds found in the island have been intentionally released as biological control agents.

Phytophagous insects are mainly sap-feeding Hemiptera (45%) and Coleoptera (24%) feeding on leaves and stems of plants (Fig. 3; Table 2). Gall formers (gallers) are mostly host-specific to alien ornamental plants, such as *Eucalyptus* spp. infested by the Australian leaf gallers *Leptocybe invasa* Fisher & La Salle, 2004 and *Ophelimus maskelli* (Ashmead, 1900) and alien *Ficus* spp. hosting a wide variety of fig wasps (Compton et al. 2020a; Demetriou et al. 2022; Demetriou et al. in press). Regarding wood-feeding insects, the only exception to Coleoptera is the Neotropical moth *Paysandisia archon* (Burmeister, 1879) (Table 2). The moth was discovered in Paphos and Limassol (Cyprus) boring in *Chamaerops humilis* L., *Phoenix roebelenii* O'Brien and *Washingtonia filifera* (Lindl.) H.Wendl. palms imported from Italy (Vassiliou et al. 2009). The infested plant material was destroyed, but due to the extended biological cycle of the insect, surveys continued until the end of the detection year (Vassiliou et al. 2009). Since then, the palm moth has been sighted once at Zygi (Larnaca) (John and Skule 2016). This demonstrates that wood boring insects can expand into new regions outside their native range even during their immature stages, which can be transported when inside their host plants (later used for planting) or even with timber (Cocquempot and Lindelöw 2010; Demetriou et al. 2021). Thus, in addition to phytosanitary measures reinforced against Hemiptera and species responsible for visually detectable infestation signs (e.g. galls, leaf mines, bite marks), monitoring imported plants and furniture for signs of infestation by wood-feeding insects constructing galleries could be informative for as a biosecurity measure.

Detritivorous species are mainly associated with household commodities and are, thus, probably introduced to the island through international commerce of stored goods. Phytophagous insects may have reached Cyprus through the introduction of their host plants, as indicated for Hemiptera (Rabitsch 2010a), but also Hymenoptera. Although introduction pathways in Cyprus are largely unknown, these assumptions are in accordance with scientific evidence pinpointing the introduction pathways of terrestrial invertebrates in Europe (Peyton et al. 2019, 2020; Pergl et al. 2020). In particular, primary pathways include stowaways and contaminants of food, plants and nursery material (Pergl et al. 2020), while secondary pathways also include the transportation of habitat material, such as soil and vegetation (Pergl et al. 2020). An extended literature survey, not only on a local, but also continental scale, would help identify the main introduction pathways of alien insects to Cyprus. Furthermore, this

information could provide information for the design of specific investigation protocols, according to the feeding habits of alien insects and the taxonomic groups present in each feeding guild, to underpin biosecurity.

Establishment status

A total of 245 alien insect species have established viable, reproducing populations on the island (Fig. 4). Species that failed to establish include the seed-beetle *Caryedon serratus*, a species considered unable to establish itself both in the wild and storehouses (Yus-Ramos et al. 2014) and the mango seed weevil *Sternochetus mangiferae* reared once from mango imported from Sri Lanka in 2011 (Biodiversity of Cyprus 2022). As stated earlier, although *Aphytis holoxanthus* was introduced to Cyprus from Israel in 1959 and 1960, it failed to establish and provide any control of *Aonidiella aurantii* (Maskell, 1879) (Gerber and Schaffner 2016).

Although reported as present in Cyprus from Burmeister (1939), the presence of the carabid beetle *Laemostenus complanatus* (Dejean, 1828) is considered unlikely (Austin et al. 2008). Despite their extensive survey work, Austin et al. (2008) failed to detect the species on the island, while it has also been stated that the species is not present in Turkey and the Middle East (Casale 1988). As explained earlier, *Rhyzobius forestieri* is also considered doubtfully present as it has not been mentioned in any literature dealing with the Coccinellidae of Cyprus and their use as biological control agents of scales (Wood 1963; Orphanides 1988; Özden et al. 2006). Lastly, records of *Oligota parva* Kraatz, 1862 and *Nomius pygmaeus* (Dejean, 1831) also seem to be doubtful as the species have been reported only from Baudi di Selve (1870) and Fauvel (1889), respectively. Since then, no records of the species have been found and their presence on the island has not been confirmed (Bordoni 2010). The only dubious record referring to Hemiptera concerns *Ploiaria chilensis* (Philippi, 1862) (Putshkov and Putshkov 1996; Rabitsch 2010b). The remaining four doubtful species are the chalcid wasp *Cirrospilus ingenuus* (mentioned above) and three ant species; *Cardiocondyla nuda*, *Trichomyrmex destructor* and *Solenopsis geminata* (Bernard 1956; Georghiou 1977; Collingwood et al. 1997; Salata et al. 2019). The alien ant fauna of Cyprus will be examined in greater detail during the following years (Demetriou et al. in prep).

Management of alien species is easier and more effective during the initial stages of biological invasion than later in the process (Simberloff et al. 2013). In Cyprus, this was the case with *Octodonta nipae*, a flower beetle which was found on young leaves of ten *Syagrus romanzoffiana* (Cham.) Glassman palms in Limassol and was rapidly eradicated (Vassiliou et al. 2011). Host-plants were potted and maintained as transplants in urban habitats of Germasogeia (Limassol), and infested plants and areas at risk (e.g. gardens, warehouses and production sites) were immediately treated with chemicals and monitored for a period of eight months "due to the long and cryptic life cycle of this palm insect pest" (Vassiliou et al. 2011). Overall, the rapid implementation of measures against *O. nipae* is considered to have resulted in successful eradication of the species on the island. The second invasive alien insect which was considered

as eradicated is the yellow fever mosquito *Aedes aegypti*, although it was predicted through horizon scanning to have a high potential for arriving again in the future (Peyton et al. 2019, 2020). However, the species was rediscovered in September 2022 at Dromolaxia, Larnaca District almost one century after it was last reported as present in the country by Aziz (1934). The presence of established populations that might have escaped past eradication efforts or the unintentional re-introduction of the yellow fever mosquito in Cyprus need to be confirmed. Nevertheless, systematic mosquito surveillance in the Akrotiri Peninsula and surrounding regions since 2012 has failed to detect the species thus far (Martinou et al. 2022a). *Aedes albopictus* (Skuse, 1894) has also been recently (October 2022) recorded at six locations in Limassol District (Martinou et al. 2022b; Christou et al. in press). Due to the most recent discovery of these invasive alien mosquito species (exceeding the data collection period), these records are not presented in our checklist, but will be added to the database.

The establishment status of 90 alien insect species (25%) is unknown, due to the collection of single specimens, incomplete record files, as well as data deficiencies in recovered, provided or investigated literature. Thus, further research is necessary to confirm the presence of these insects on the island. Material sampling and identification of alien species in museum and personal collections, as well as communication with experts and digitalisation of grey literature could assist these endeavours. For example, little is known about the establishment status of alien Phthiraptera, where all species were catalogued as "unknown". These data deficiencies could be addressed through the construction and maintenance of databases with observations from veterinarians and municipal veterinary services. Knowledge gaps also appear in Coleoptera, Hemiptera and Lepidoptera where the establishment status was considered as unknown for 38%, 29% and 23% of cases, respectively.

Intentional introductions – Biological control agents

Releases of alien insects as biological control agents in Cyprus reached a peak during the 1960s (Fig. 5; Suppl. material 2). During this decade, half of the released classical biological agents were introduced to the island, to tackle the increased damage caused to cultivations, particularly due to citrus pests, such as the hemipterans *Chrysomphalus aonidum* (Linnaeus, 1758), *Lepidosaphes beckii* and *Planococcus citri* Risso, 1813 (Wood 1963; Gerber and Schaffner 2016). These releases concerned the Asian ladybugs *Chilocorus circumdatus* (Gyllenhall, 1808) and *Chilocorus hauseri* Weise, 1895 whose impacts and establishment are unknown, as well as the import of representatives of the genus *Aphytis* Howard, 1900 (Wood 1963). Although alien Aphelinidae seemed to offer at least partial control of their associated pests, range expansion of *Aphytis melinus* DeBach, 1959 and *A. coheni* led to the competitive exclusion of native-to-Cyprus *Aphytis chrysomphali* (Mercet, 1912) (Orphanides 1984). Ichneumon and braconid releases during the 1960s were aimed at controlling populations of *Phthorimaea operculella* (Gerber and Schaffner 2016). Most species established viable populations on the island although their efficacy as biological control agents remains unknown (Gerber and Schaffner 2016).

Releases during the 1980s included that of *Comperiella bifasciata* Howard, 1906 against the citrus pest *Aonidiella aurantii* (Orphanides 1996), as well as the African species *Metaphycus helvolus* (Compere, 1926) and *Metaphycus lounsburyi* (Howard, 1898) successfully combating the olive grove pest *Saissetia oleae* (Orphanides 1993). In the 1990s, four additional chalcid wasps were recruited against the citrus leaf miner *Phyllocnistis citrella* Stainton, 1856 (Schauff et al. 1998), although their overall impact is rather unknown (Gerber and Schaffner 2016). The reported intentions of rearing and release of the Neotropical chalcid wasp *Cales noacki* Howard, 1907 against *Aleurothrixus floccosus* (Maskell, 1896) have been confirmed (EPPO 1997; Nicos Seraphides pers. comm.). Since the beginning of the 21st century, no data on intentional releases of alien biological control agents were found. Although this may show that alien insects have not been imported to the island during the last two decades, the presented data (Fig. 5) may also indicate the lack of published information in scientific journals.

The history of biological control agents in Cyprus is largely intertwined with commercial potato, olive and citrus crops. Most of the intentionally introduced species have successfully established on the island (81%). However, the efficacy of these releases remains unknown for the vast majority of species (71%). Out of the 19 alien species introduced against citrus pests, only four were reported to offer some degree of control over their hosts (Gerber and Schaffner 2016). Regarding potato crops, out of the ten alien biological control agents introduced to the island, only *Apanteles subandinus* was reported to effectively tackle the common and destructive potato moth *Ph. operculella* (Greathead 1976; Georghiou 1977; Gerber and Schaffner 2016). The inaccessibility of data may be the reason why the percentage of biological control agents considered to be successful in controlling the pest, against which they were released, is seemingly low. For example, in contrast to the only data made available online, stating the intentions of introducing and rearing *C. noacki* (EPPO 1997), the species has not only been introduced and released, but it is also well-established and has provided successful control of *A. floccosus* (Nicos Seraphides pers. comm.).

It could be informative to map the current distribution of historically-known introduced biological control agents to the island, such as *Aphelinus mali* (Haldeman, 1851) or *Copidosoma koehleri* Blanchard, 1940, species for which the presence of established populations is currently unknown (Gerber and Schaffner 2016). The presence, efficacy and non-target effects of biological control agents would be valuable for assessing the benefits of these species, while documenting overall impact towards native biodiversity.

Impacts

Positive impacts of alien insects are largely anecdotal. Out of the 37 species identified, less than half (41%) concerned intentional introductions of biological control agents, as the efficacy of most intentionally introduced species remains unknown. The remaining insects, unintentionally introduced along with their host-plants, reduce the growth of alien invasive plants, such as that of *Leucaena leucocephala* (Lam.) de Wit by its obligate seed-feeding beetle *Acanthoscelides macrophthalmus* (Schaeffer, 1907) (Vassiliou

and Papadoulis 2008) or *Ficus microcarpa* L. suppressed by alien non-pollinating fig wasps that inhibit its seed-germination and subsequent spread (Demetriou et al. in press). Although the 37 insect species with registered positive impacts may be presumed as beneficial, the overall lack of studies assessing their integration into natural ecosystems and food-chains may bias such conclusions.

Studies addressing the adverse impacts of alien insects in Cyprus mostly focus on insects of agricultural or horticultural significance (Morris 1937; Georghiou 1977; Kontodimas et al. 2006; Şişman and Ülgentürk 2010; Ülgentürk et al. 2015; Compton et al. 2020b; Hadjiconstantis and Zoumides 2021). The impacts of alien insects on the biodiversity of Cyprus has received minimal attention, evidenced by the small number of alien insects (19 species) classified as invasive (Fig. 6). These species include, *inter alia*, the rosemary beetle *Chrysolina americana* infesting native aromatic plants (Hadjiconstantis and Zoumides 2021), the aphid *Myzus persicae* Sulzer, 1776 (Georghiou 1977; Ioannou and Iordanou 1987), as well as two scale insects *Aspidiotus nerii* Bouché, 1833 and *S. oleae* feeding on native and alien plants (Morris 1937; Georghiou 1977; Orphanides 1993; Şişman and Ülgentürk 2010; Compton et al. 2020b). In addition, four hymenopterans are known to compete with and displace native species (Orphanides 1984; Salata et al. 2019), while another has been found parasitising a native scale insect (Georghiou 1977). Lastly, two alien Siphonaptera, *Ctenocephalides canis* (Curtis, 1826) and *Ctenocephalides felis* (Bouche, 1835) have been found to negatively affect human and animal health as vectors of pathogens (Psaroulaki et al. 2006, 2014) (Suppl. material 2).

The impacts of alien insects in Cyprus are unquestionably in need of a detailed literature investigation covering both published and unpublished literature. Impact assessments, using the protocols and criteria of EICAT and SEICAT (Hawkins et al. 2015; Bacher et al. 2017; IUCN 2020; Kumschick et al. 2020a), to comprehensively assess the impacts of alien insects of Cyprus would be valuable in providing a list of invasive alien species of national concern. Additionally, such impact assessments could be included within risk assessments and should take into account any recorded impacts of alien insects studied in neighboring Mediterranean and Middle-Eastern countries (e.g. Egypt, Greece, Israel and Turkey). Thus, these assessments could act as an early warning system for insects with harmful impacts detected in neighboring regions (Kumschick et al. 2020b), which have been classified as data-deficient in Cyprus, but also species remaining undetected or yet to have reached the island. Nevertheless, the updated checklist of alien-to-Cyprus insect species (Suppl. material 1) constitutes an important first step towards prioritising management decisions and implementing monitoring schemes for invasive alien species on the island.

Horizon scanning for invasive alien species with the potential to threaten biodiversity, human health and the economy of Cyprus (including insects) have been already implemented, addressing species with high likelihood of arrival, establishment and potential impacts (Peyton et al. 2019, 2020). A total of 14 alien insects likely to be imported and established on the island were prioritized. Amongst them, four alien mosquitoes (*Aedes* spp.) with the potential to harm human health and well-

being, crop-pests, such as *Leptinotarsa decemlineata* Say, 1824, *Daktulosphaira vitifoliae* (Fitch, 1855) and *Anoplophora* spp., but also invasive alien species, such as *Linepithema humile* (Mayr, 1868) and *Vespa velutina* Lepeletier, 1836 (Peyton et al. 2019, 2020). These species could be included in alert-lists (Peyton et al. 2019). Management measures could benefit from the construction of dynamic, dichotomous identification keys available online for the rapid identification of species in order to support border control and phytosanitary surveillance. In parallel, data availability, usefulness and transparency could be enhanced by data-digitisation of grey literature, following core biodiversity data standards (Groom et al. 2017). In accordance with these recommendations, the updated checklist and data curated by the ACE database are being integrated to the CyDAS, with hopes that they can assist risk assessments on a national and European level.

Lastly, the large percentage of alien insects assessed as data-deficient (47%) (Fig. 6) combined with the high percentage of species whose establishment status is considered unknown or presence is doubtful (25%) (Fig. 4), clearly illustrate the necessity for "more boots in the ground" (Wilson 2017) regarding the study of insects in Cyprus.

Conclusions

Since 2010, the number of documented alien insect species known to inhabit Cyprus has tripled. A total of 349 alien species have been detected while there are already a number of new additions to the checklist. Updated resources including identification keys are needed to raise awareness and support biosecurity strategies.

As introduction pathways of alien insects in Cyprus are largely unknown, stowaways and contaminants of food and plants could be prioritised, since they comprise the most common introduction pathways in Europe (Pergl et al. 2020). Future endeavours include deciphering the biological invasion history, distribution, impacts and species interrelationships of alien insects by utilising classical methods, citizen science and molecular tools. Data and studies focusing on alien insects will be also fed to larger databases, such as the CyDAS, GRIIS and GBIF, to ensure data interoperability (Penev et al. 2021).

Regarding intentional introduction and release of alien biological control agents, educational material and information on the taxonomy, history and efficacy of imported biological control agents could be made available online to the public and scientific community. Such information could be catalogued to register and monitor both importers and providers of biological control agents in order to keep track of alien species intentionally released on the island. The ACE and CyDAS databases can act as data repositories ensuring the accumulation, availability and transparency of data on alien species assisting monitoring and further research efforts, risk assessments, prioritisation of invasive alien species, management strategies and lastly, the establishment of rapid response/early warning systems mitigating further introductions and impacts of invasive alien species.

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Supplementary material 1

Checklist of alien insects of Cyprus

Authors: Jakovos Demetriou, Canella Radea, Jodey M. Peyton, Quentin Groom, Alain Roques, Wolfgang Rabitsch, Nicos Seraphides, Margarita Arianoutsou, Helen E. Roy, Angeliki F. Martinou

Data type: checklist

Explanation note: Checklist of alien insects of Cyprus. Legend: Status = Alien (A), Cryptogenic (C), or Questionable (Q); Establishment status = Established (Es), Failed to establish (Fa), Introduced but failed to establish (In), Eradicated (Er), Doubtful (Do) and Unknown (Un).

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Supplementary material 2

Alien biological control agents intentionally introduced to Cyprus

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Data type: database

Explanation note: Data on alien biocontrol agents intentionally introduced to Cyprus including their taxonomy, introduction year/period/decade, origin, establishment status, host (reason of import), impact, and reference(s).

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RESEARCH ARTICLE

NeoBiota

Escape from the garden: spreading, effects and traits of a new risky invasive ornamental plant (*Gaillardia aristata* Pursh)

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Abstract

Ornamental plants constitute a major source of invasive species. *Gaillardia aristata* (great blanketflower) is planted worldwide and its escape has been reported in several European countries without ecological impact assessment on the invasive potential. As there is a markedly spreading population with invasive behaviour in Hungary, we aimed to reveal the distribution, impacts and traits of *G. aristata*. We gathered occurrence data outside the gardens in Hungary, based on literature, unpublished observations by experts and our own records. We investigated the impacts of an extended population, where the species invaded sandy old-fields within a 25 km² area. Here, we compared the species richness, diversity, community composition and height of invaded and uninvaded vegetation. Furthermore, we evaluated the traits potentially associated with the invasiveness of *G. aristata* in comparison with other herbaceous invasive species in the region. We found that *G. aristata* occurred mostly by casual escapes, but naturalised and invasive populations were also detected in considerable numbers. *G. aristata* usually appeared close to gardens and ruderal habitats, but also in semi-natural and natural grasslands and tended to spread better in sandy soils. We found lower plant species richness and Shannon diversity in the invaded sites and the invasion of *G. aristata* significantly influenced the composition of the plant community. The trait analyses revealed that the invasive potential of *G. aristata* is backed by a wide germination niche breadth, extremely long flowering period, small shoot-root ratio (large absorption and gripping surface), large seeds (longer persistence) and dispersal by epizoochory of grazing livestock (mostly by sheep), probably helping the species' survival and

spreading in the disturbed, species-poor, sandy, open habitats. These functional traits, as well as the ornamental utilisation, may act together with the aridisation of the climate and the changing land-use practices (e.g. abandoned, disturbed sites) in the success of *G. aristata*. We raise awareness of the rapid transition of *G. aristata* from ornamental plant to casual alien and then to invasive species in certain environmental conditions (i.e. sandy soils, species-poor communities, human disturbances), although it seems to be not a strong ecosystem transformer so far. Nonetheless, banning it from seed mixtures, developing eradication strategy and long-term monitoring of this species would be important to halt its spreading in time.

Introduction

Unintentional and intentional human activities have the greatest role in the introduction of new species (Hulme et al. 2008; Gallien and Carboni 2017). Species introduced as a commodity (e.g. ornamental or garden plants) constitute a major source of potentially invasive species (Auer 2008). These species are artificially relocated into a new area with human help, where the required environmental conditions (e.g. watering, nutrition, pesticide control) are assured (Haeuser et al. 2019). Through these, introduced ornamental plants are able to pre-adapt to the local abiotic conditions, enhancing their chance to escape (Richardson et al. 2000; Hulme et al. 2008; Marco et al. 2010). After a potential escape, they face the barriers of the naturalisation-invasion process (Richardson and Pyšek 2012), while climatic and environmental factors, as well as functional traits of invasive species, act together to influence their success and ability to disperse (Dietz and Edwards 2006; Gallien and Carboni 2017; Haeuser et al. 2017). However, not all non-native species can survive and co-exist with the resident native species in a long term (Gallien and Carboni 2017).

Dozens of ornamental plants have been introduced from warmer climatic regions, such as the low-latitude regions of North and South America (Haeuser et al. 2019). Thus, by global warming, they will probably encounter soon the required environmental conditions on a larger scale (Bradley et al. 2010; IPCC 2021), increasing their chance of escaping and possibly becoming invasive species (Haeuser et al. 2017, 2019; Fahey et al. 2018; Cao et al. 2021). Amongst functional traits, long flowering period, large height, seed mass and total biomass, high germination rate and dispersal ability, as well as high stress tolerance, were proved to enhance the invasion potential of a plant species (Nentwig 2007; Richardson and Pyšek 2012; Gallien and Carboni 2017; Wang et al. 2018). These characteristics are also typical for the species used in horticulture because they facilitate plant establishment and growth in gardens (Marco et al. 2010). A new generation of invasive ornamental plant species is to be expected, which requires the attention of ecologists and conservationists (Bradley et al. 2010). Therefore, early warning systems are needed (Rainford et al. 2020), which monitor the escaped

ornamental plants, present regional invasion events and assess the impact of non-native species at an early stage (Hulme 2006). These actions may help to alert other countries and to detect and blacklist immediately these species (Seebens et al. 2017).

Gaillardia species were introduced as ornamental plants to Europe in the 18th century (Stoutamire 1960). The great blanketflower (*Gaillardia aristata* Pursh), a native species in North America is planted worldwide (Wiersema and León 2013). It is classified mostly as weedy or naturalised species, with a medium-level weediness rating by Randall (2017). Its spread has been reported in more than 10 European countries (Randall 2017; Roy et al. 2020) and it is reported as invasive in Russia (Vinogradova et al. 2010). In Hungary, the first escape of this species was observed in the early 20th century (Soó 1954; Priszter 1960). Balogh et al. (2004) classified it as a naturalised species, while a decade later, Korda et al. (2018) labelled it as dangerous and not recommended for planting in Hungary. In 2019, we surveyed an extended population near Izsák (Bács-Kiskun County, mid-Hungary; Molnár et al. (2003)) with invasive behaviour and considerable abundances across 25 km² (see Fig. 1). Due to the missing knowledge about the species' ecological impact and invasion ecology, here, we aimed to obtain information about the invasiveness of this ornamental plant species and to raise awareness.

The objectives of this study were: 1) to map the occurrence of *G. aristata* in Hungary, 2) to study the potential impacts of *G. aristata* on the vegetation and 3) to understand its invasiveness through its traits in comparison with other herbaceous non-native invasive plant species of the invaded region. For objective 1), we gathered all available information on the establishments outside the gardens from literature and experts, including our own observations. For objective 2), we compared the species richness, diversity, community composition and height of the old-field vegetation between sites invaded by *G. aristata* and their uninvaded control pairs along a coverage gradient of *G. aristata* at the above-mentioned single location. For objective 3), we compared the traits of *G. aristata* and other non-native invasive herbaceous species present in the study region to understand the role of different biological attributes in its invasive behaviour.

Figure 1. An extended population of *Gaillardia aristata* near Izsák, Hungary (46.791434, 19.298135) with invasive behaviour.

Materials and methods

Studied species

G. aristata is a perennial herb belonging to the Asteraceae family (Hegstad and Maron 2019), native to North America (Winslow 2011b). It is a typical species of short-grass prairie (Winslow 2011a) and prefers dry, open habitats with a Mediterranean climate (Randall 2017) due to its drought tolerance and adaptability to well-drained soils (Winslow 2011a). Its mean height is around 65 cm (Winslow 2011b). In its native range, it germinates early (April/May), blooms from the end of spring with a long flowering period and bears fruit from summer to autumn (Hegstad and Maron 2019; Kattge et al. 2020), with relatively large achenes and long hairy pappus (Winslow 2011b). The seeds' properties indicate anemochory and epizoochory (Chytrý et al. 2021), although many seeds fall directly beneath the maternal plant (Hegstad and Maron 2019). It does not reproduce with clonal spreading (Kattge et al. 2020), but can re-seed in abundance (Winslow 2011b). It is a mid-successional species establishing dense populations in disturbed areas in its native range (Taylor 1992; Winslow 2011b). A wide variety of pollinators visit *G. aristata* for pollen and nectar in its native range (Winslow 2011b) and also in Central Europe (Kovács-Hostyánszki et al. 2022).

Occurrence of *G. aristata* within Hungary

We started to map the spontaneous distribution of *G. aristata* outside the gardens in Hungary after we became aware of the invasive population in our studied area (see the next "Study site" section). We gathered the occurrence data from literature, personal communications of experts and our own records. To find current occurrences, we used the "Distribution atlas of vascular plants of Hungary" database (Bartha et al. 2022). During literature scanning, we used Arcanum Digitheca (https://www.arcanum.com/en/), MATARKA (Hungarian Periodicals Table of Contents Database, https://matarka.hu/) and ad hoc literature scanning. We contacted field experts to collect new occurrence data of this species and recorded our own observations in 2018–2022. We scanned online databases, i.e. iNaturalist (https://www.inaturalist.org/) and Pl@ntNet (https://plantnet.org/) and marked occurrences indicating high probability of escapes (i.e. the occurrence was not in a garden, while in herbaceous vegetation). We have not recorded localities where the species could be just planted out to the street front, for example, the population showed signs of care including well-kept or weeded out surroundings, regular shape etc. However, in the case of ornamental plants, it is not easy to determine whether an occurrence within a settlement is the result of human planting or spontaneous escape.

We presented the occurrence map of *G. aristata* using qGIS software (QGIS 2022). We considered the approx. 6.25 km × 5.55 km grid of Central European mapping grid system (CEU; Ehrendorfer and Hamann (1965)) for the dataset of Bartha et al. (2022)

and marked all the gathered localities of *G. aristata*. We gathered the habitat types of occurrences according to the Ecosystem Map of Hungary (http://alapterkep.termesze-tem.hu/) with 20 m resolution (Agrárminisztérium 2019; Tanács et al. 2021), using qGIS. The 20-metre resolution of the Ecosystem Map of Hungary conceals some details, i.e. the exact grassland type in the urban areas. In the future, recording the exact invaded habitat type is highly recommended. Furthermore, we grouped the recently documented occurrences as: 1) casual escape, 2) naturalised and 3) invasive (based on Richardson et al. (2000)).

## Invasion effects of *G. aristata*

Study site

The location of our study sites was near Izsák City, Hungary (Fig. 2B). The landscape was covered by small-scale, mostly sandy old-fields between scattered homesteads due to the declining agricultural activity (Molnár et al. 2003). A military facility has operated in this region since the 19th century (Honvédség 1897; Mesznéder 2005), which heavily influenced the natural vegetation with soil disturbances and frequent fire events (Molnár et al. 2003). According to a local pensioner, *G. aristata* was planted around the former Matyó Castle (Izsák: Matyódűlő) in the 1950–60s and was transplanted from there to other gardens, from where it escaped. Molnár et al. (2003) reported the first record of its spread at the border of Izsák, without major conservation concern at that time (Sipos 2004). Today, *G. aristata* occurs in all kinds of habitats, except forests and wetlands in the neighbourhood including former croplands, but also natural and semi-natural open and closed pannonic sand steppic grasslands with the risk of further spreading (Fig. 2; Suppl. material 1). We sampled only this small locality because, in the year of sampling (2019), only our study sites near Izsák were known as invaded areas by an extended population of *G. aristata* with high abundance.

Botanical sampling

We sampled 50 m × 50 m habitat patches in seven pairs of sandy old-fields in a 5 km × 5 km landscape window in June 2019 (Fig. 2B). One of the site pairs was invaded by *G. aristata* ("invaded sites", > 10% *G. aristata* (absolute) coverage), while the other one was uninvaded ("control sites", absent or < 3% *G. aristata*). Site pairs were close to each other (median: 613 m, min–max: 189–1481 m distance between the pairs) and had similar habitat conditions.

We estimated the percent green cover of all herbaceous plant species within three 3 m × 3 m plots randomly placed within the 50 m × 50 m habitat patches. Plant species were identified by prior knowledge and by field guides (Simon 2000; Király 2009) and we used plant names according to World Flora Online (WFO 2022). We also measured the height of the local vegetation at 10 random points in all plots.
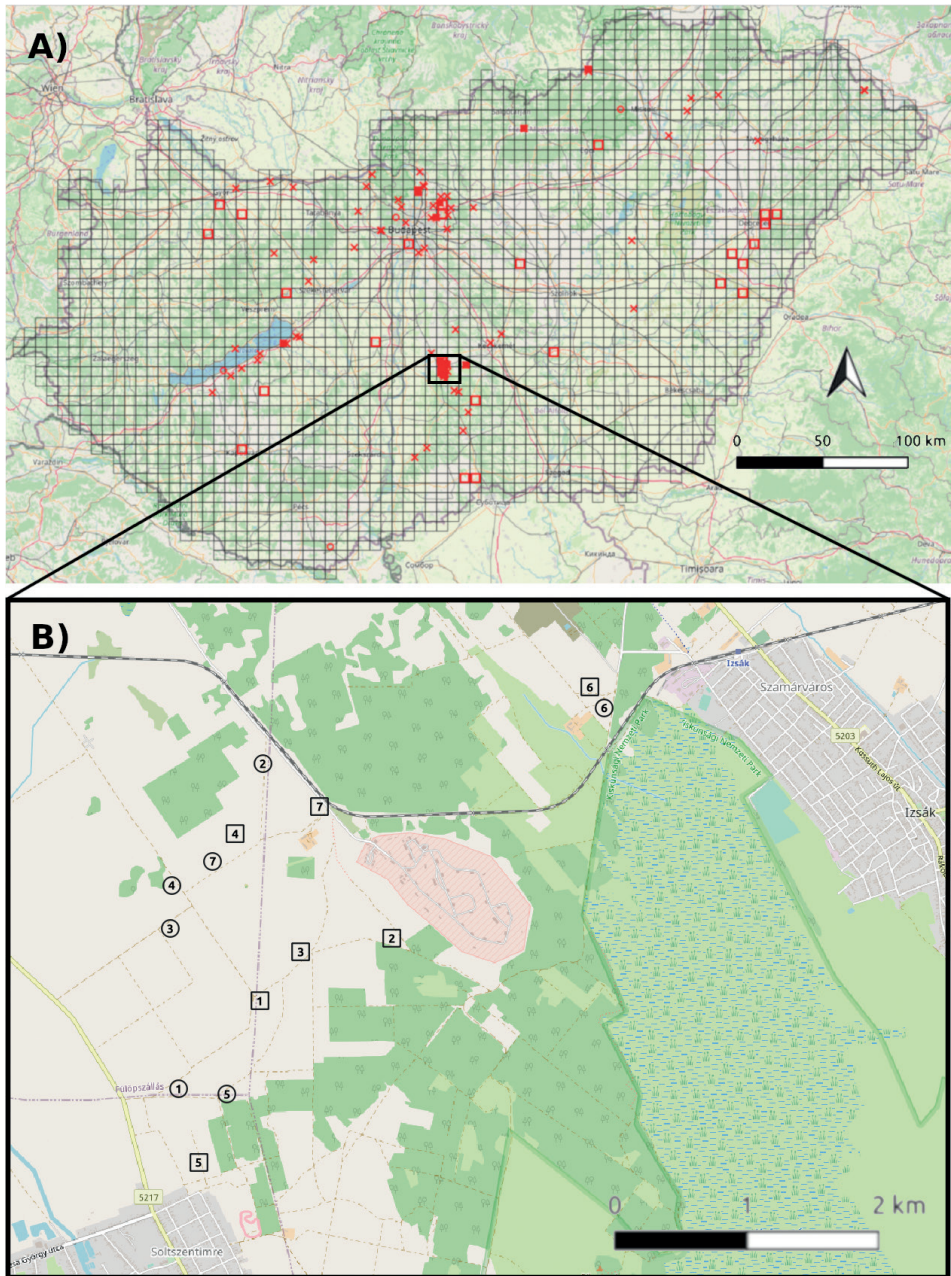
Figure 2. The occurrence of *Gaillardia aristata* within Hungary (**A**) and the sampling sites (**B**) **A** red squares represent the occurrences from the "Distribution atlas of vascular plants of Hungary" database (Bartha et al. 2022) in approx. 35 km² grid of "Mapping of the Central European Flora" (shown by black thin grid). Red circles represent historical data from literature. Red crosses represent casual escape, while red filled squares represent naturalised or invaded populations, based on data from online databases, own observations and personal communications by experts. We do not present three points where the species has disappeared (see details in Suppl. material 1) **B** squares represent the sampled invaded, while circles represent control sites for invasion ecology study of *G. aristata*. Numbers represent site pairs. Map data 2022 OpenStreetMap.

Statistical analyses

We analysed the data at plot-level. We obtained the species richness of plants from the cumulative number of species for each plot without *G. aristata*. We calculated Shannon diversity both including and subtracting the cover values of *G. aristata* for each plot. In this way, we differentiated between *G. aristata*'s contribution to and impact on the diversity of local plant communities (Thomsen et al. 2016). We also calculated the average of the 10 vegetation height values for each plot.

First, we analysed the differences in species richness, the two types of Shannon diversity (henceforth, the inclusion and subtraction models, respectively) and vegetation height between the invaded and control sites. The explanatory variable was the status of the sites (i.e. invaded vs. control sites). Second, we analysed the effect of *G. aristata* coverage on the species richness, Shannon diversity indices and the height of vegetation. The explanatory variable was the percentage cover of *G. aristata*. We applied generalised linear mixed models (GLMMs) with Poisson distribution for species richness and with Gaussian distribution for Shannon diversity indices and vegetation height (Venables and Ripley 2002; Zuur et al. 2009). Site pairs were built into the model as random factors. We adjusted p-values separately for response variables using the method of Benjamini and Hochberg (1995). We calculated pseudo-R^2 (Nakagawa and Schielzeth 2013) for all models.

Furthermore, we analysed the effects of invasion and the coverage of *G. aristata* on the community composition of plants with and without *G. aristata*. We applied Permutational Multivariate Analysis of Variance (PERMANOVA), using Bray-Curtis dissimilarities for species-level percentage data (Borcard et al. 2018). We included the sampled site pairs as "blocks" (i.e. random factor) to handle the non-independencies in the sampling structure. We adjusted p-values separately for response variables using the method of Benjamini and Hochberg (1995). We also performed non-metric multidimensional scaling (NMDS; Borcard et al. (2018)) to reveal the pattern of the coverage of *G. aristata* in the plant communities. We presented the first two dimensions of NMDS. We generated smooth surfaces along the coverage gradient of *G. aristata* with generalised additive models (GAMs) to interpolate the fitted values on the NMDS plot. We also evaluated the association of plant species (without *G. aristata*) to invaded or uninvaded sites by indicator species analysis (Borcard et al. 2018). The indicator values of the species were tested via the Monte-Carlo simulation using 10,000 permutations. The accepted significance level was $p < 0.05$.

The statistical analyses were carried out using the R v.3.6.3 statistical environment (RCoreTeam 2020), "glmmTMB" v.1.1.2.3 and "performance" v.0.8.0 packages for GLMMs (Brooks et al. 2017; Lüdecke et al. 2021), "vegan" v.2.5-6. for PERMANOVA (Oksanen et al. 2019) and "labdsv" v.2.0-1 packages for indicator species analysis (Dufrêne and Legendre 1997).

Traits of *G. aristata* and non-native herbaceous invasive species

In order to understand the invasion mechanism of *G. aristata*, its biological traits were measured. To interpret the invasiveness of this species, by descriptive statistics, its traits

were compared to other herbaceous non-native invasive species from our studied region (based on Balogh et al. (2004)): *Ambrosia artemisiifolia* L.; *Asclepias syriaca* L.; *Erigeron annuus* (L.) Pers.; *E. canadensis* L.; *Helianthus tuberosus* L.; *Impatiens glandulifera* Royle; *Oenothera villosa* Thunb.; *Phytolacca americana* L.; *Reynoutria japonica* Houtt.; *Solidago canadensis* L.; *S. gigantea* Aiton; *Xanthium strumarium* subsp. *strumarium*. The chosen non-native invasive species are highly successful in the studied region, but their success is backed by different trait syndromes and ecological strategies. We might identify crucial traits with outstanding trait values that define the possible invasive strategy of *G. aristata* by comparing them along the traits usually associated with invasiveness (van Kleunen et al. 2010). For this (and other purposes), we collected traits of East European invasive plants for a comprehensive trait database (Fenesi et al., unpublished data). This is the nearest, available database which contains a series of functional traits of all important herbaceous invasive plants of the region.

To test the optimal timing of germination for each species, we collected seeds and fruits ("seeds" for the sake of simplicity) of these species from invasive populations in Transylvania, Romania in the summer and autumn of 2020, from at least 30 individuals of one population, mixed and kept in paper bags. We calculated the germination rate (%) for all invasive species in three germination conditions (autumn, early spring and late spring). In the temperate climate of Europe, plant species' seeds germinate in autumn (species with seeds without dormancy, for example, many annuals, biennials or species of disturbed habitats) or in spring (species that need a short or long chilling period to break the seeds' dormancy; Walck et al. (2011)). Therefore, we simulated these conditions to offer the species the possibility to show how wide their germination niches are. Seeds were put to germinate in autumn, after dry-storage for one-two months and in spring, after cold-wet stratification in the dark at temperature of 1–4 °C for three months. Five replicates of 20 seeds were placed in plastic Petri dishes filled with two filter papers for each treatment. The sealed dishes with fresh seeds were put in germination chambers (Sanyo MLR-352H; Sanyo/Panasonic Healthcare Co., Ltd, Japan.) in October 2020, simulating autumn conditions (11 hours light at 15 °C, 13 hours darkness at 5 °C); while stratified seeds were placed in germination chambers in April 2021, simulating early spring conditions (13 hours light at 15 °C and 11 hours darkness at 5 °C) or late spring conditions (14 hours light at 20 °C and 10 hours darkness at 10 °C). The Petri dishes were regularly watered with distilled water and monitored three times a week; all germinated seeds were recorded and removed.

To present the average height (cm) and the beginning and duration of flowering (month) of invasive species, we gathered the data on the minimum and maximum height and the flowering phenology from a Hungarian field guide (Király 2009). For *G. aristata*, we used Flora of North America (Strother 2020). Most of the biennials and perennials did not flower in the pots during our one vegetation season experiment, so we could not use these data.

To calculate the shoot-root ratio, total biomass (g) and specific leaf area (mm²/mg), seeds were put to germinate in 1-litre pots, filled with potting soil. The emerging seedlings (one per pot, ten replicates for each species) were allowed to grow exactly eight weeks after the first true leaves were observed. The pots were watered twice a week with the same

amount of water, depending on the weather conditions: more in sunny and warm weeks and less on rainy and cloudy days. We intended to set up an optimal water condition, i.e. not just to wet the soil on the surface, but to give enough water to the whole pot. This meant about 80% of field capacity. The experiment took place in an open-air facility with transparent roof in the University Botanical Garden in Cluj-Napoca, Romania. After two months of growth, shoots with leaves and roots were separated for five plants per species, washed and dried in an oven for 48 h at 65 °C and were weighed to calculate shoot-root ratios and total biomass. Three-five plants were allowed to grow till maturity and served to calculate specific leaf area (SLA, leaf area per unit leaf mass, mm^2/mg) for each species. We collected three-five mature, but non-senescent leaves from each individual. Leaf area was calculated based on photographs of leaves using ImageJ software (Abràmoff et al. 2004). Leaves were dried for 48 h at 65 °C and weighed.

Seed mass (g) was obtained by weighing three sets of 100 seeds from each species, using an analytical scale (Kern ABJ 80- 4NM, with 0.1 mg resolution). We calculated the average weight of one hundred seeds.

The terminal velocity (m/s) of the seeds, i.e. the maximum rate of fall, expressed the wind-dispersal ability. We measured the duration of seed descent and we divided the height of fall (1.47 m) by the duration of fall by the methods of Andersen (1992). The lowest value of terminal velocity expresses the highest ability of seeds to be dispersed by wind (Ruprecht et al., unpublished data).

Epizoochory (%) was tested as the likelihood of seeds attaching to sheep fur. We focused on sheep due to their likely presence in the studied landscape and to be a possible vector of propagules. We followed the protocol of Moravcová et al. (2010): a wooden frame was covered with sheep fur and the fur was pressed to a plain surface with seeds spread over it. After three circular movements of the frame pressed to the surface, the number of propagules attached to the fur was counted. For each species, four replicates of 25 propagules, thus 100 propagules altogether were involved. Please note: terminal velocity and epizoochory are available only for 10 from the 13 investigated species.

Results

Occurrence of *G. aristata* within Hungary

For *G. aristata*, we gathered 119 occurrences altogether in 89 (3%) approx. 35 km^2 CEU cells all over Hungary. We found 27 records (26 CEU cells) in the database of Bartha et al. (2022), four historical records in four CEU cells (Soó 1954; Priszter 1960) and five occurrences (five CEU cells) from online databases (Fig. 2A; Suppl. material 1). We could supplement these data with 83 recently documented records (54 CEU cells) by personal communications of experts and own observations (Fig. 2A; Suppl. material 1). The habitat types of *G. aristata* occurrences were urban area in 28.6% (incl. green urban area with/without trees, low building, other paved or non-paved artificial area, paved road, railway), semi-natural herbaceous vegetation in 27.7% (incl. closed/open sand steppe, closed grassland in hills and mountains, salt steppe and

meadow, other herbaceous vegetation), cropland in 10.9% (incl. arable land, vineyard, complex cultivation pattern with/without scattered buildings), woodland in 4.2% (incl. black locust-dominated mixed plantation, other ligneous vegetation, woodland) and unknown in 28.6% of the total number of occurrences. The occurrences were casual escapes in 55.5%, naturalised in 6.7%, invasive population in 16.8% and unknown in 21.0% of the total number of occurrences. The "unknowns" refer to disappeared populations and the lack of exact coordinates in literature or the online database.
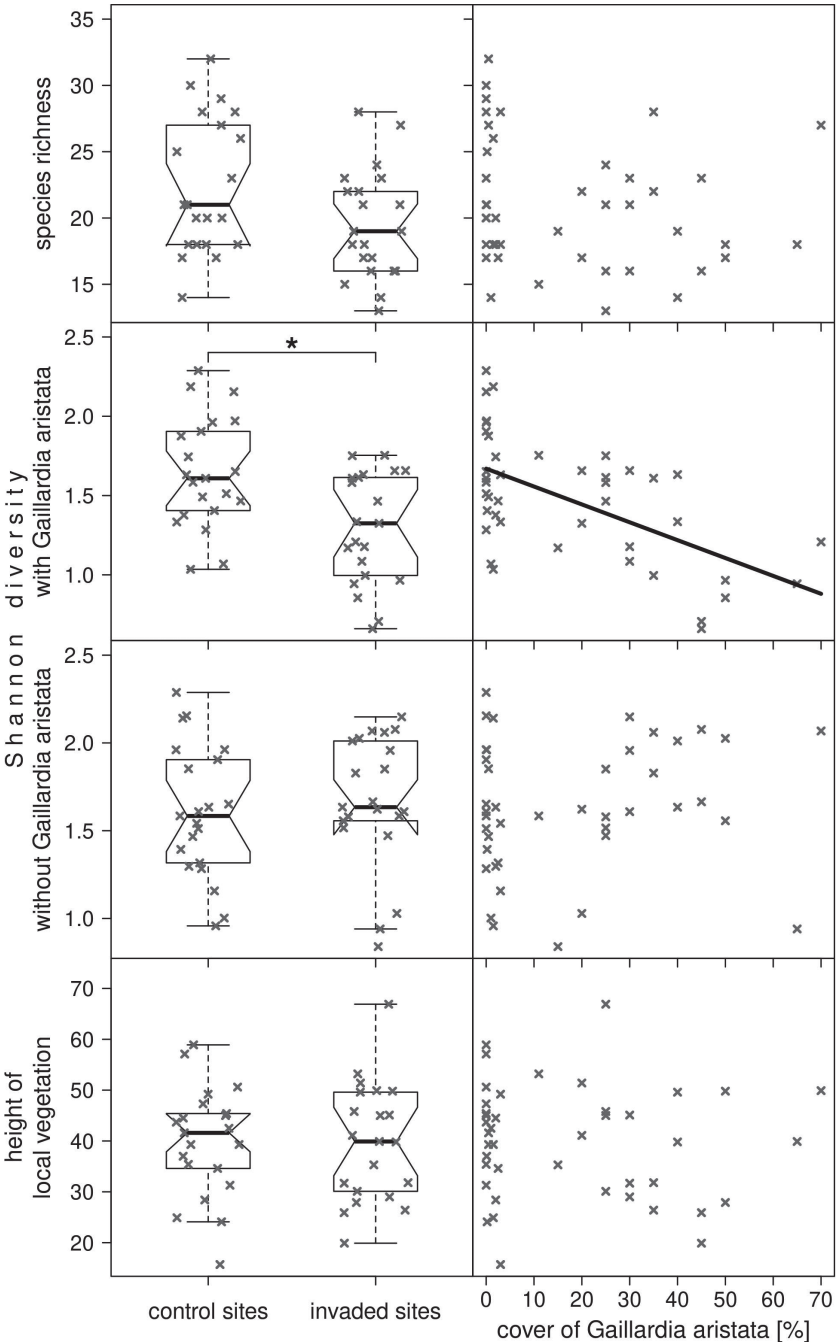
Invasion effects of *G. aristata*

We recorded 110 plant species in total in our study sites, 23–45 (min–max) species per site (without *G. aristata*). The average height of the local vegetation was 39.8 cm in both the invaded and control sites. The plots of invaded sites were covered 11–70% (mean: 34.8%) by *G. aristata*. We found slightly lower plant species richness in invaded sites compared to the controls (adjusted p-value = 0.080; Fig. 3; Suppl. material 2). We did not find any effect of invasive coverage on the plant species richness (adjusted p-value = 0.095; Suppl. material 2). Shannon diversity was significantly lower in the invaded sites (adjusted p-value = 0.008) and decreased with increasing *G. aristata* cover in the inclusion models (adjusted p-value < 0.001). On the contrary, there was no significant difference (adjusted p-value = 0.530) between the invaded and control sites and the cover of *G. aristata* had no significant effect on Shannon diversity in the subtraction models (adjusted p-value = 0.530). There was no significant difference between the control and invaded sites in the height of local vegetation (adjusted p-value = 0.996) and the cover of *G. aristata* had no significant effect on the vegetation height (adjusted p-value = 0.996; Fig. 3; Suppl. material 2).

We found significant differences in community composition between invaded and control sites in the inclusion models (adjusted p-value < 0.0001; R² = 0.25) and also in the subtraction models (adjusted p-value = 0.0004; R² = 0.07; by PERMANOVA; Fig. 4). Furthermore, we found that the cover of *G. aristata* had a significant effect on the community composition in the inclusion (adjusted p-value < 0.0001; R² = 0.23) and in the subtraction models (adjusted p-value = 0.0006; R² = 0.07; by PERMANOVA; Fig. 4). The analysis of indicator species regarding invasion showed that four species were linked to invaded and eleven species to control sites (Table 1).

Traits of *G. aristata* and non-native invasive herbaceous species

Compared to the other more common non-native invasive herbaceous species in the region, *G. aristata* had an outstandingly high germination ratio both in autumn and during spring, showing a wide germination niche breadth (Fig. 5). Its flowering period was also extremely long and started very early compared to other species (Fig. 5). Regarding its stature, it is the shortest plant amongst the studied invasive species, thus its biomass was also small, but it invested considerably more energy in roots compared to aboveground parts. Seeds were proved likely to be dispersed by animals and less likely by wind (Fig. 5).

Figure 3. Effects of invasion (control vs. invaded) and cover of *Gaillardia aristata* on species richness, Shannon diversity (with and without *G. aristata*) and the height of local vegetation. Box plots show medians, lower and upper quartiles, notches show 95% confidence intervals. Grey × symbols represent sampling plots. Significant differences (after p-value adjustment) between the invaded and control sites are indicated by star (*) above the boxes and the significant effect of invasion cover by continuous lines according to the GLMMs (see Suppl. material 2).
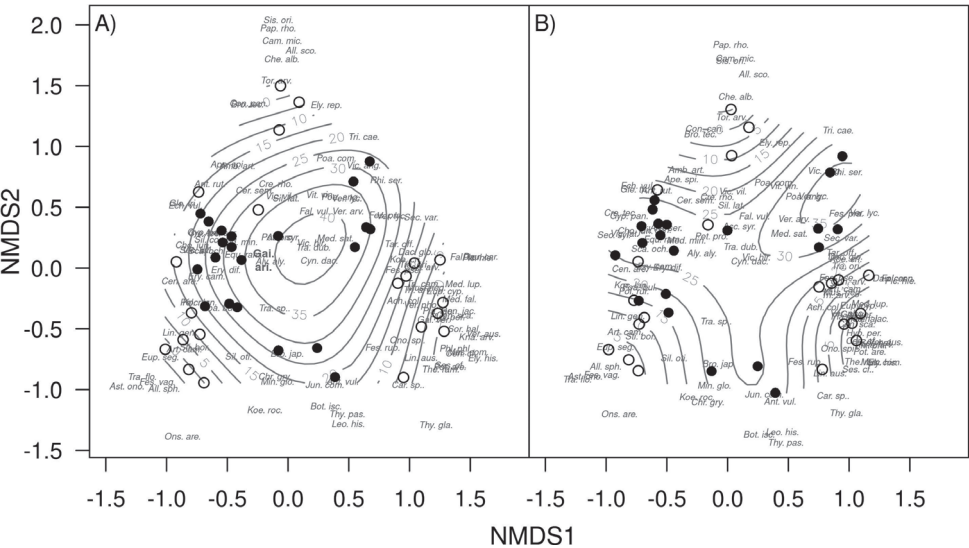
Figure 4. Community composition by NMDS ordination **A** including *Gaillardia aristata* and **B** excluding *G. aristata*. Filled circles represent the plots of invaded sites, while empty circles are the control sites. GAM fitted isoclines represent cover percentages of *G. aristata*. The italic abbreviated names indicate the plant species. *G. aristata* is highlighted by bold and larger font size on **A** part of the Figure.

Table 1. The results of analysing indicator species regarding *Gaillardia aristata* invasion. The accepted significance level was $p < 0.05$.

Species	p-value	IndVal	Control	Invaded
Control sites				
Centaurea scabiosa L.	0.001	0.473	0.476	0.048
Crepis foetida subsp. *rhoeadifolia* (M.Bieb.) Čelak.	0.036	0.556	0.571	0.381
Cynodon dactylon (L.) Pers.	0.002	0.749	0.810	0.571
Erigeron canadensis L.	0.026	0.332	0.333	0.143
Euphorbia cyparissias L.	0.004	0.381	0.381	0.000
Euphorbia seguieriana Neck.	0.046	0.271	0.286	0.095
Festuca rupicola Heuff.	0.016	0.398	0.429	0.143
Festuca vaginata Waldst. & Kit. ex Willd.	0.019	0.285	0.286	0.048
Galium verum L.	0.018	0.368	0.381	0.095
Plantago lanceolata L.	0.034	0.413	0.429	0.190
Securigera varia (L.) Lassen	0.009	0.333	0.333	0.000
Invaded sites				
Chondrilla juncea L.	0.008	0.457	0.143	0.571
Medicago sativa L.	0.043	0.271	0.048	0.286
Secale sylvestre Host.	0.021	0.457	0.238	0.476
Vicia villosa Roth	0.038	0.475	0.238	0.571

Discussion

Ornamental plants pose a great risk of escaping and turning into invasive species under human disturbances and climate change (Auer 2008; Hulme et al. 2008; Bradley et al. 2010; Klonner et al. 2019). *G. aristata* is a widely planted ornamental species, its
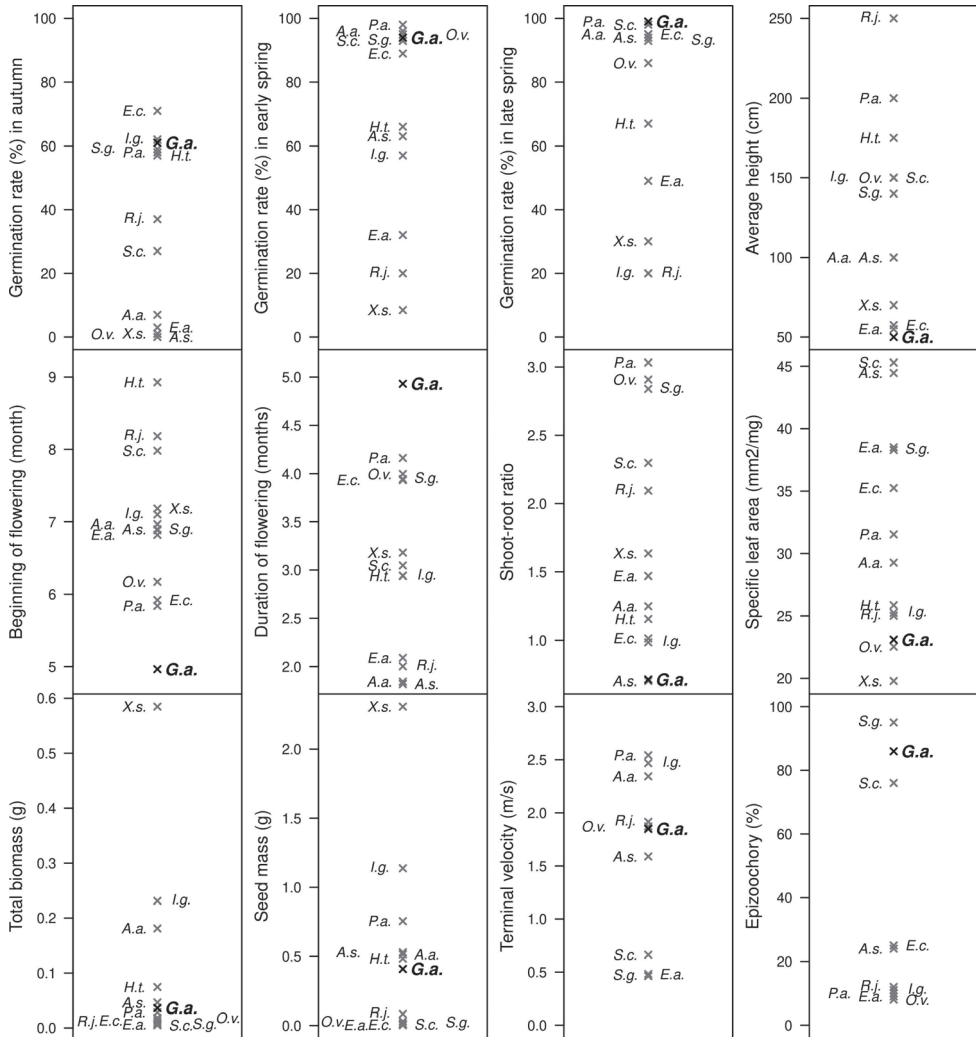
Figure 5. Traits distribution of the non-native invasive herbaceous species. The trait comparison between *Gaillardia aristata* (*G. a.*) and other invasive herbaceous species in the region: *Ambrosia artemisiifolia* (*A. a.*); *Asclepias syriaca* (*A. s.*); *Erigeron annuus* (*E. a.*); *E. canadensis* (*E. c.*); *Helianthus tuberosus* (*H. t.*); *Impatiens glandulifera* (*I. g.*); *Oenothera villosa* (*O. v.*); *Phytolacca americana* (*P. a.*); *Reynoutria japonica* (*R. j.*); *Solidago canadensis* (*S. c.*); *S. gigantea* (*S. g.*); *Xanthium strumarium subsp. strumarium* (*X. s.*). The x-axes only present the abbreviations of the investigated species, while the × symbols represent the exact trait values along the y-axes.

escape and naturalisation having been repeatedly reported before (Molnár et al. 2003; Randall 2017; Korda et al. 2018; Roy et al. 2020), but its invasive behaviour is relatively unknown (except in Russia; Vinogradova et al. (2010)). Meanwhile, in the past few years, this species presented a rapid transition from naturalisation to invasion in Hungary. Therefore, our aim was to document the circumstances of this invasion phenomenon by assessing the ecological impact of *G. aristata*, in order to raise awareness of a potential future spread in Eurasia as well.

Occurrence of *G. aristata* within Hungary

G. aristata was found in 89 (3%) approx. 35 km² CEU cells all over Hungary, based on the former (26 cells by Bartha et al. (2022) and four cells by historical data from literature) and recently documented (59 cells; including online databases) occurrence data. *G. aristata* occurred mostly in sandy and other loose textured soils, usually close to gardens and in other man-made or ruderal habitats. However, it was also observed in natural and semi-natural sandy grasslands and even in humid loess grasslands. There were localities where it escaped and spread, but then declined or disappeared from one year to the next due to building construction (e.g. in Erdőkertes; own observation) or without any known reason (e.g. in Soroksár Botanical Garden; Mária Hőhn ex lit.). In many locations, it was present sporadically for years, but was not able to spread aggressively (own observations). In many places (e.g. on roadsides), it was probably maintained by human disturbances (Taylor 1992; Winslow 2011b). Invasive populations were about 17% of the occurrences that might be potential hotspots of further spread to nearby native habitats. As well, there is the threat that any naturalised population or even some casual escapes may be able to transform into invasive.

Other *Gaillardia* species (Indian blanketflower (*G. pulchella* Foug.) and their hybrid (*G. × grandiflora* Van Houtte)) also escape from gardens and spread in Europe (Randall 2017). Together with *G. aristata*, they are challenging to identify due to similar phenotypic characteristics and their highly variable flowers (Hammond et al. 2007; Lengyel 2022). However, the three taxa differ slightly in their life cycles (Stoutamire 1960) and, while the other two taxa show only casual escapes in Hungary (personal observation; Bartha et al. (2022)), *G. aristata* escapes more often, establishing naturalised and invasive populations and seems to spread vigorously.

The impact of *G. aristata* on invaded plant communities

In our studied population, *G. aristata*'s invasion had only a moderate negative impact on the sampled old-field vegetation, suggesting only a slight potential inhibition on the distribution and growth rate of local species, as well as some potential changes in succession (Levine et al. 2003; Csecserits et al. 2011; Powell et al. 2011; Pyšek et al. 2012). Based on our results, this species is not a strong ecosystem transformer in old-fields. However, invasive species are able to slow down or halt secondary succession hindering the recovery towards semi-natural grassland vegetation (Cramer et al. 2008; Fenesi et al. 2015). The cover of an invasive species can often explain its effects on the local vegetation (Hejda et al. 2009). Moderate cover in general (such as in the case of *G. aristata*) usually results in a minor effect on the plant community compared to non-native plants with high coverage (Csecserits et al. 2011; Albert et al. 2014). However, even moderate invasion could affect other related communities, for example, pollinators. Comparing the same invaded and non-invaded old-fields, *G. aristata* had a significant effect on the abundance and diversity of hoverflies, on the abundance of honeybee and on the community composition of floral resources (Kovács-Hostyánszki et al. 2022).

The community composition and indicator species analyses of invaded and non-invaded sites suggested three different types of non-invaded old-field. Such differences can be originated from the initial seed bank, land-use history, time since abandonment as arable land, current management and the process of succession (Inouye et al. 1987; Csecserits et al. 2011). The indicator species for control sites, such as *Festuca rupicola* Heuff., *F. vaginata* Waldst. & Kit. ex Willd. and *Secale sylvestre* Host., are the dominant grass species of the closed, the perennial open and the annual open sandy grasslands (Borhidi et al. 2012), respectively, indicating two target grassland communities and a disturbed annual association during old-field succession. The invaded sites were in an intermediate phase in species composition between the mentioned grassland types (Borhidi et al. 2012). Based on these species characteristics, our study was carried out on different types of species-poor old-fields, indicating also the influence of successional pathways on the community composition. This difference amongst study sites may be an outcome of the higher beta diversity within control sites compared to more homogenised invaded sites (Socolar et al. 2016), reducing the opportunity to detect differences in diversity and in community composition.

It has to be acknowledged that this was an observational and not an experimental study; hence, we were only able to take a snapshot of the invasive behaviour of *G. aristata*. Furthermore, our study covered a relatively small area at a given location compared to the country- (and even continent-)wide distribution and the potential long-term changes of this species. We cannot rule out the possibility that the chosen control and invaded sites differed in some aspects before the invasion (e.g. differences in land-use), facilitating the spread of *G. aristata* (Davis et al. 2005). However, we did our best to choose sites with similar site conditions as possible and we assumed that the control and invaded sites differed only in the presence of *G. aristata*. The presence of this species is relatively new to the region, as well as *G. aristata* spreading as a frontline in the studied region (pers. comm. of Csaba Bíró, the Ranger of the National Park near Izsák). Here, *G. aristata* occurs in all kinds of habitats, except forests and wetlands in the neighbourhood. Based on our field experiences, we hypothesise that the possible differences between the sites may not exclude the possibility of invasion, just influence the abundance of this species in the invaded areas and the timing of invasion for the not yet invaded dry ecosystems. Nevertheless, the impact of *G. aristata* was found not really hazardous compared to the general effects of the real transformer invasive plant species (Vilà et al. 2011; Qi et al. 2014). However, an extended study would be needed to investigate the long-term effect of *G. aristata*, which might become stronger (e.g. *A. syriaca*; Csecserits et al. (2016)), stagnant or weaker (e.g. *A. artemisiifolia*; Csecserits et al. (2009)) during succession.

Traits and environmental conditions drive the invasion

According to our knowledge, our study area is the first location where the invasive behaviour of *G. aristata* has been studied. Therefore, the drivers behind the invasive mechanism are important to understand. We aimed to find out how this potentially

invasive species might be similar to other, more successful invasive species in this region and which traits of *G. aristata* might explain its invasion and success in certain habitats. *G. aristata* germinates early with resistance to allelopathic chemicals (Tyrer et al. 2007). Meanwhile, it can re-seed even in the same season (own field observation) due to its wide germination niche breadth and the mild autumn weather by the warming climate (Haeuser et al. 2017). Its chance of survival is further increased by its drought tolerance and adaptability to well-drained soils (Winslow 2011b). *G. aristata* has low SLA, which can help to maintain plant water status during drought, increasing its drought tolerance (Nautiyal et al. 2002; Girdthai et al. 2010). The small shoot-root ratio of this species indicates significant allocation to roots compared to aboveground parts of the plant, which provides large absorption and gripping surface (Noordwijk and Willigen 1987), facilitating the colonisation and spreading in loose-textured soils, such as sand. The species has no known allelopathic effect (Kattge et al. 2020), but *G. aristata* probably grows faster and taller (see Fig. 5) than most of the local species in sandy vegetation (see the average vegetation height in control sites). Thus, *G. aristata* may inhibit or delay the growth of seedlings of other species by shading and reducing water availability (Levine et al. 2003). In contrast, the larger seeds of this species (Hegstad and Maron 2019), which is non-typical for invasive plants (Rejmánek and Richardson 1996; Radny et al. 2018), could be disadvantageous in spreading. However, it can disperse well with epizoochory (Baltzinger et al. 2019), probably by sheep as well as by the main cattle species in the study area (Molnár et al. 2003). Thus, it has a continuous local seed rain with a slow, but steady seed spread-rate (Kattge et al. 2020) along with the possibility that large seeds are able to sustain the seedling under poor conditions with sufficient nutrient tissue (Jakobsson and Eriksson 2000).

G. aristata was in lag phase for several decades (Pyšek and Hulme 2005), but now it certainly encountered its required environmental conditions in this area (Haeuser et al. 2017, 2019) by climate change (i.e. warmer, arid climate (Winslow 2011b)) and human disturbances (Bradley et al. 2010; IPCC 2021) facilitating its spreading (Haeuser et al. 2019; Klonner et al. 2019). Land-use changes (i.e. land abandonment) produce suitable species-poor and disturbed habitats for this mid-successional species (Davis 2009; Winslow 2011b). Additionally, *G. aristata*'s cover was increased after wildfire events in its natural habitat (Antos et al. 1983), the short-grass prairie (Winslow 2011a) that is heavily controlled by fire (Wright and Bailey 1981). In our study region, the frequent fire events by military activities (i.e. firing range and training ground) and the potential grazing of abandoned old-fields by sheep (Molnár et al. 2003) may explain why *G. aristata* has been able to spread and shows invasive behaviour in this area. Besides these habitat conditions, also people and pollinators may admire and help to sustain this ornamental plant, due to its beauty, long flowering period and resources as a bee pasture (Lindemann-Matthies 2016; Kovács-Hostyánszki et al. 2022).

In summary, the climatic and environmental factors (aridisation of the climate and dry, nutrient-poor, rapidly warming sandy surfaces), the land-use (abandoned, probably burned arable fields), the competitive functional traits (drought tolerance, long flowering period, large roots and seeds) and the ornamental utilisation of *G. aristata* seem to act together to influence its success and ability to disperse and to become a

new, dangerous invader in dry, species-poor habitats (Molnár et al. 2003; Dietz and Edwards 2006; Gallien and Carboni 2017; Haeuser et al. 2017; Korda et al. 2018). However, uncovering the details behind a species changing into an invasive is worth further investigations. For example, revealing the differences between invasive and casual populations in traits (e.g. chemical composition; Cappuccino and Arnason (2006)) and environmental parameters (e.g. soil type) and investigating the further effect of climate change on the spreading (Mojzes et al. 2020; Orbán et al. 2021).

Conservational recommendations

For all newly-established non-native species, monitoring, ecological impact assessments and also experiments on eradication should be required (Gallien and Carboni 2017). For *G. aristata*, it would be important to carry out eradication experiments to investigate the detailed invasion biology and the opportunity to inhibit the further spreading (Hulme et al. 2008; Bradley et al. 2010). As *G. aristata* does not reproduce clonally (Kattge et al. 2020), ploughing the invaded area and then over-seeding it with a seed mixture of native species probably can be an appropriate eradication strategy in old-fields (Holt 2009). However, the longevity and persistence of *G. aristata* seeds in the seed bank are unknown. If the seed bank of *G. aristata* is persistent, it will probably remain for years (as for example, *A. artemisiifolia* Milakovic and Karrer (2016)). Hence, both the seed longevity and the seed bank persistence in the field, as well as the effect of the different eradication methods need further studies. Grazing is a questionable potential solution (Holt 2009; Winslow 2011b). Light grazing did not affect *G. aristata* (Daubenmire 1970), while it decreased by vigorous sheep and increased by cattle grazing in its original place of residence (Mueggler and Stewart 1980). However, heavy grazing is not a suitable conservation treatment for dry grasslands (Deng et al. 2014; Molnár et al. 2020); moreover, the grazing animals could play a role in its spreading in the neighbourhood. Hence, exploring the effectiveness and indirect influences of different grazing and mowing regimes is necessary.

The example of *G. aristata* spectacularly identifies that one of the most common sources of plant invasion is ornamental planting (Auer 2008; Pyšek et al. 2017). The European Union maintains a blacklist of invasive species (European Parliament 2014); however, it includes only a few species, which may require regular revision and misses species that are only later found to be problematic (Seebens et al. 2017). A list of discouraged ornamental plants, including frequent escapers and aggressive spreaders, would be needed to raise the attention of gardeners at least. The sale of *G. aristata* alone or in ornamental seed mixtures should be banned in Eurasia, especially in regions with sandy habitats.

Conclusion

In this study, we investigated the occurrences, ecological impact and traits of a new, risky, invasive, ornamental plant species, *G. aristata*. This species escaped and spread in disturbed, semi-natural and natural habitats. Although the invasion of *G. × grandiflora*

has already been observed in Belgium (Branquart et al. 2007; Verloove et al. 2020), it seems that in Central Europe *G. aristata* also crosses the threshold of invasion and may start to spread in the East European steppe and further. Our work demonstrated only a moderate negative impact on the old-field vegetation; so far, *G. aristata* is not a strong ecosystem transformer. Besides reviewing its phenological and morphological traits supporting the invasive behaviour, some questions remained unanswered; thus further thorough studies are needed to evaluate the exact causes behind its transformation into invasive. More attention should be paid to *G. aristata*, because there is a chance of spreading and becoming invasive elsewhere in certain (i.e. sandy, species-poor, disturbed) conditions. This ornamental species is planted and nursed in many locations (e.g. gardens, parks and facilities); hence, it can escape from many more places in the future, while people also contribute to the invasion process (Lindemann-Matthies 2016). Thus developing effective eradication strategy and long-term, continuous, systematic mapping within Eurasia would be important to reveal and halt the spreading of *G. aristata* (Hulme 2006; Royimani et al. 2019; Papp et al. 2021).

Acknowledgements

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Supplementary material 1

Occurrence of *Gaillardia aristata* within Hungary

Authors: Gabriella Süle, Zsombor Miholcsa, Csaba Molnár, Anikó Kovács-Hostyánszki, Annamária Fenesi, Norbert Bauer, Viktor Szigeti

Data type: table (Xlsx file)

Link: https://doi.org/10.3897/neobiota.83.97325.suppl1

Supplementary material 2

The results of mixed models analysing the effects of the invasion and the coverage of *Gaillardia aristata* on the species richness, Shannon diversity and the height of local vegetation

Authors: Gabriella Süle, Zsombor Miholcsa, Csaba Molnár, Anikó Kovács-Hostyánszki, Annamária Fenesi, Norbert Bauer, Viktor Szigeti

Data type: table (Docx file)

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RESEARCH ARTICLE

Invasive terrestrial invertebrate detection in water and soil using a targeted eDNA approach

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Abstract

Terrestrial invasive invertebrates can rapidly colonise new areas, causing detrimental effects on biodiversity, economy and lifestyle. Targeted environmental DNA (eDNA) methods could constitute an early detection tool given their sensitivity to small numbers of individuals. We hypothesised that terrestrial runoff would transport eDNA from the land into adjacent waterbodies and used the invasive yellow crazy ant (*Anoplolepis gracilipes*) as a model species to test this hypothesis. We collected water samples from four waterbodies adjacent (< 10 m from the creek edge) to infestations following rainfall events for eDNA analysis. We also collected soil samples from areas of known infestations and tested five eDNA extraction methods to determine their efficiency to extract eDNA from soil. Water samples resulted in positive yellow crazy ant eDNA amplification (20–100% field replicates across all sites), even at one site located 300 m away from where ants had been detected visually. Soil samples resulted in a higher percentage of false negatives when sampled from ant transit areas than from nest entrances. Unpurified DNA extracts from soil also resulted in false negative detections and only after applying a purification step of DNA extracts, did we detect yellow crazy ant eDNA in 40–100% of field replicates across all methods and sites. This is the first study to empirically show that eDNA from a terrestrial invertebrate can be successfully isolated and amplified from adjacent or downstream waterbodies. Our results indicate that eDNA has the potential to be a useful method for detecting terrestrial invertebrates from soil and water.

Keywords

Introduction

Over the past 15 years, environmental DNA (eDNA) analysis has gained momentum for biomonitoring of both marine and freshwater aquatic systems (Zaiko et al. 2018; Sepulveda et al. 2020; Trujillo-González et al. 2021). Targeted-eDNA methods (i.e. quantitative real-time polymerase chain reaction [qPCR]-based) are now considered a sensitive, specific and robust tool for detection of aquatic or semi-aquatic invasive species (Ficetola et al. 2008; Piaggio et al. 2014; Smart et al. 2015; Villacorta-Rath et al. 2020). This has resulted in eDNA methods being increasingly adopted into monitoring programmes by natural resource management agencies, consultancy companies and citizen-science groups (Darling and Mahon 2011; Larson et al. 2020). However, advances in the use of eDNA to detect aquatic species have not been matched by developments for terrestrial species.

A key challenge in using targeted eDNA as a method to detect terrestrial species is determining an effective sampling strategy (Taberlet et al. 2012; Van Der Heyde et al. 2020). This needs to consider where eDNA is likely to be deposited by the target organism, the effect of the substrate on eDNA detectability and ease of sampling. To date, a few studies have used soil samples for targeted eDNA detection (Kucherenko et al. 2018; Katz et al. 2021; Yasashimoto et al. 2021) and most of them have reported false negatives arising from sampling soil areas that the species did not occupy (Kucherenko et al. 2018; Yasashimoto et al. 2021). Similarly, DNA binds to soil to a varying level depending on its physicochemical composition and inappropriate eDNA extraction methods can lead to qPCR inhibition, affecting eDNA detectability and data interpretation (Andersen et al. 2012; Katz et al. 2021; Yasashimoto et al. 2021).

Invasive ants are amongst the most harmful invasive species globally (Kenis et al. 2009; Siddiqui et al. 2021); they threaten the environment, human health and livelihoods and social amenities (Holway et al. 2002; Lach and Hooper-Bùi 2010; Gruber et al. 2022). Millions of dollars are spent on prevention, treatment and control of ant invasions globally to avoid these impacts (Zenni et al. 2021). Current methods for invasive ant detection (i.e. baited traps or cards, pitfall traps and detection dogs) rely on luring, trapping, smelling or sighting active individuals or colonies, which can be labour-intensive, costly and reliant on species behaviour and weather conditions (Hoffmann et al. 2010; Lach and Barker 2013; L. Lach, unpublished data). These methods have low detection sensitivity to low numbers of individuals, which can lead to false negatives (Stringer et al. 2011; L. Lach, *pers obs.*). Environmental DNA analysis could improve detectability of invasive ants, since it does not require sighting the target species (Jerde et al. 2011). However, studies investigating terrestrial insect eDNA capture for species detection are seldomly available and are designed in agricultural contexts, wherein their sampling strategy consisted of spraying water over crops to aggregate the available eDNA deposited there (Valentin et al. 2018, 2020; Allen et al. 2021).

In this study, we test different methods to capture and detect terrestrial invertebrate eDNA. We use the yellow crazy ant, *Anoplolepis gracilipes* (Smith, 1857), one of

the most environmentally and socioeconomically damaging invasive insect species in the world (Clarke et al. 2021; Gruber et al. 2022), as a model species. We collected water and soil samples in field conditions to: (1) investigate whether yellow crazy ant eDNA can be detected from creeks or rivers adjacent to existing infestations; and (2) to compare the efficiency of laboratory extraction methods for eDNA from soil samples. We hypothesised that terrestrial runoff would transport eDNA from the land into adjacent waterbodies.

Materials and methods

Study system

The yellow crazy ant is a widespread invader in tropical regions, particularly the Indo-Pacific (Janicki et al. 2016). Colonies consist of multiple interacting nests, usually termed 'supercolonies' (Abbott 2005; Hoffmann 2014). In forested areas, nests are typically at the base of trees or under rocks, leaf litter or logs, but the ants will nest virtually anywhere with the right temperature, humidity and protection from sunlight (e.g. discarded car engines and soft drink cans) and can readily relocate nests when disturbed (Hoffmann 2015; Lach, pers. obs.). Workers may also shelter temporarily in nest-like sites without brood or queens, for unknown periods of time (Lach, pers. obs.). Their nesting and foraging behavioural plasticity make choosing appropriate sites from which to sample soil for eDNA more challenging than it may be for other species, such as Argentine ants, *Linepithema humile* (Yasashimoto et al. 2021). The first recorded infestation of the yellow crazy ant in mainland Australia was in the Northern Territory in the early 1990s (Majer 1984). Yellow crazy ant incursions have been reported in Queensland, Australia, since 2001, including in the Cairns and Townsville regions (Lach and Hoskin 2015).

Townsville is in the "dry tropics" region of Australia and is characterised by a wet (November to April) and a dry (May to October) season. During the wet season, ambient temperature ranges between 23.2 °C (± 1.4) and 30.9 °C (± 0.7), there is a mean humidity of 65.6% (± 5.1) and a mean rainfall of 169.3 mm (± 102.7) (http://www.bom.gov.au/climate/averages/tables/cw_032040.shtml). During the dry season, ambient temperature ranges between 16.6 °C (± 2.6) and 27 °C (± 1.6), the mean humidity is 58.2% (± 5.7) and mean precipitation is 19.9 mm (± 8.6) (http://www.bom.gov.au/climate/averages/tables/cw_032040.shtml).

### Environmental DNA sample collection

Water samples for eDNA analysis were collected and preserved from waterbodies adjacent (< 10 m from the creek edge) to yellow crazy ant infestations in Townsville, Queensland, Australia, with "high activity" (M. Green, Townsville City Coun-

cil, pers. obs.) in February and March 2021 (Ross River, unnamed creek adjacent to Chauncy Crescent, Stuart Creek) (Table 1, Fig. 1), during or immediately after rainfall events. We also sampled from an unnamed creek (Palmetum site) in which yellow crazy ants had been detected 300 m from the creek edge (M. Green, Townsville City Council, pers. obs.). At each site, five replicate 30 ml surface water samples were collected using a sterile 50 ml Falcon tube and decanted into another 50 ml Falcon tube containing 10 ml of Longmire's preservative solution (Longmire and Baker 1997) as per Villacorta-Rath et al. (2021). Replicate water samples were taken approximately 10 m apart. At every site, a field blank was also carried out to ensure that the process of sample collection did not introduce contamination. The field blank consisted of decanting 30 ml of laboratory-grade water into a Falcon tube containing 10 ml of preservative solution.

Soil samples were collected during two other sampling events in Townsville at known infestation sites (Table 1, Fig. 1). Soil samples were not collected at the same time as water samples because ants tend to retreat into their nests during rainfall events, when water samples were collected. Soil sampling took place in the morning and dusk and between the months of December and April, when, due to the environmental conditions of the sampling region, yellow crazy ant activity is likely to be high (Hoffmann 2015). During the first sampling event (December 2020; Table 1), samples were collected from areas where ants could be observed transiting (hereafter referred to as ant transit samples). Seven replicate samples were collected from two sites along 50–70 m transects, starting where ant activity was observed. One of the sites (Gieseman Road site; Table 1, Fig. 1) was adjacent (< 10 m away) to a water body (Stuart Creek, Table 1, Fig. 1). Sampling consisted of collecting 2 ml of soil into a sterile 50 ml Falcon collecting tube. Subsequently, 1 ml of this soil was transferred

Table 1. Field sites where water and soil samples were collected for eDNA analyses in the Townsville area, north Queensland. All samples were collected by the authors, except for water samples from Stuart Creek, which were collected by the Townsville City Council.

Site	Latitude, Longitude	# eDNA replicates	Collection date	Collection time	Sample volume per replicate
Water eDNA samples					
Ross River	19.3127°S, 146.7563°E	5	26/02/2021	morning	30 mL
Creek at Chauncy Crescent	19.3126°S, 146.7575°E	5	26/02/2021	morning	30 mL
Creek at Palmetum	19.3114°S, 146.7631°E	5	26/02/2021	morning	30 mL
Stuart Creek	19.3405°S, 146.8533°E	5	8/03/2021	morning	30 mL
Soil eDNA samples – Ant transit sampling event					
Gieseman Road	19.2680°S, 146.5784°E	7	10/12/2020	morning	1 g
Copper Refinery	19.3411°S, 146.8530°E	7	10/12/2020	morning	1 g
Soil eDNA samples – Nest entrance sampling event					
Gieseman Road	19.2680°S, 146.5784°E	10	27/04/2021	morning	1 g
Douglas	19.3126°S, 146.7575°E	10	26/04/2021	dusk	1 g
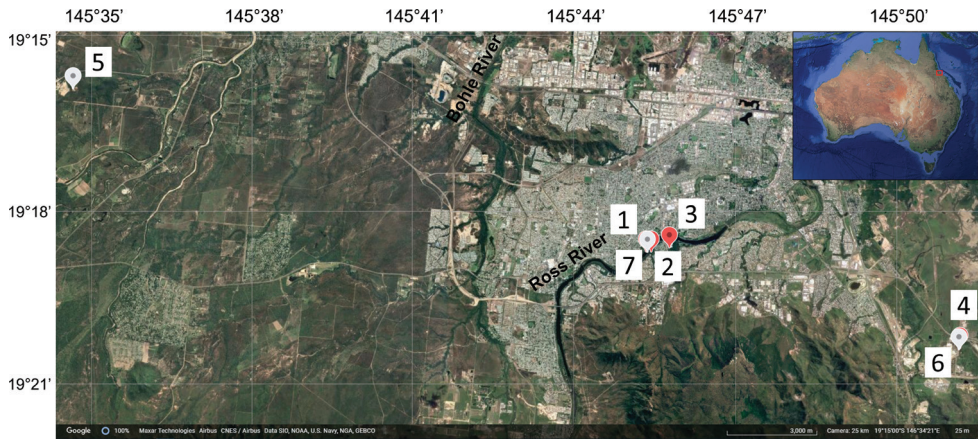
Figure 1. Sampling sites where eDNA samples were collected in the Townsville region, Queensland, Australia. Water sampling sites included: (1) Ross River; (2) creek at Chauncy Crescent; (3) creek at Palmetum; and (4) Stuart Creek. Soil sampling sites included: (5) Gieseman Road; (6) Copper Refinery; and (7) Douglas. Map Data: Google 2023 CNES / Airbus, Maxar Technologies.

into a tube containing 700 µl cetyltrimethylammonium bromide (CTAB) buffer from the CTAB protocol (Adamkewicz and Harasewych 1996) and the other 1 ml into a tube containing 2 ml of Biomeme lysis buffer from the M1 Bulk Sample Prep Kit for DNA – High Concentration (Philadelphia, Pennsylvania). Two field controls were collected to assess potential field contamination: one was a tube containing 700 µl CTAB buffer and the other was a Biomeme homogenisation tube containing 2 ml of Biomeme lysis buffer.

In the second sampling event (April 2021; Table 1), we collected soil from suitable microhabitats (bases of trees and under fallen logs) that had visible evidence of high yellow crazy ant traffic and high incidence of dead ants, which were considered putative nest entrances (hereafter referred to as ant nest entrance samples). In this case, ten 50 ml replicate soil samples were collected from two sites. One of the sites (Gieseman Road, Table 1) was resampled; however, the other previously sampled site (Copper Refinery) had recently been treated with insecticide, so samples were collected from a different site (Douglas: Table 1). Similar to the Gieseman Road site, the Douglas site was adjacent (< 10 m away) to a water body (Ross River, Table 1). Sampling consisted of collecting the equivalent of 50 ml of soil in sterile plastic containers, carefully avoiding visible dead ants. Containers were stored in ice and immediately transported to the laboratory at James Cook University. Each sample was then shaken to homogenise and approximately 1 g of soil was partitioned into four sub-samples for extraction method comparisons and stored in DNA LoBind 2 ml tubes at -80 °C until eDNA was extracted. Field controls consisted of five tubes containing the lysis buffers of each eDNA extraction method (described below).

DNA extraction, quantitative real-time polymerase chain reaction and sequencing

Environmental DNA extraction from water

We extracted eDNA from water samples at the dedicated TropWATER eDNA laboratory, James Cook University, Townsville, Australia. We followed the preserve, precipitate, lyse, precipitate and purify (PPLPP) method in Edmunds and Burrows (2020). Briefly, the PPLPP workflow uses a glycogen-aided isopropanol-based precipitation, followed by a guanidinium hydrochloride and TritonX-based lysis and a subsequent glycogen-aided polyethylene glycol (PEG)-based precipitation. For each eDNA extraction batch, an extraction control was added to ensure that no contamination was introduced during laboratory procedures. This consisted of decanting 30 ml of laboratory-grade water into a Falcon tube containing 10 ml of preservative solution in the laboratory. DNA extracts were then purified to remove environmental inhibitors using the Qiagen DNeasy Power-Clean Pro Cleanup Kit (Germantown, Maryland), as per the usual procedure of eDNA water sample analysis (Villacorta-Rath et al. 2020; Villacorta-Rath et al. 2021).

Environmental DNA extraction from soil

Ant transit soil samples were extracted using two methods: the field-based extraction and the laboratory-based method. The field-based extraction method involved using the Biomeme M1 Bulk Sample Prep Kit for DNA – High Concentration (Philadelphia, Pennsylvania) and the laboratory-based method involved using a chloroform-based extraction protocol (CTAB; Adamkewicz and Harasewych (1996)). For the field-based extraction, the equivalent of 1 ml of soil sample was added to a Biomeme homogenising tube with 2 ml of Biomeme lysis buffer and mixed vigorously using vortex mixing for one minute. After this time, the supernatant was drawn from the tube using the Biomeme extraction column and syringe and pumped out 20 times. Extraction methods were then followed as per the manufacturer's instructions and eluted in 100 µl of Biomeme Elution buffer. For the laboratory-based extraction, the equivalent of 1 ml of soil sample was directly transferred into a microcentrifuge tube containing 700 µl CTAB buffer and 10 µl proteinase K was added to samples upon arrival to the laboratory following Adamkewicz and Harasewych (1996). Samples were vortexed, crushed with plastic pestles and left to lyse at 65 °C in a hibernation oven on a rocking platform for 20 hours. After lysis was performed, 700 µl chloroform-isoamyl was added and samples were centrifuged at 16,000 g for 10 min. The supernatant was then transferred into a new tube containing 600 µl chloroform-isoamyl and centrifuged again at 16,000 g for 10 min. The resulting supernatant was transferred into a new tube containing 600 µl of cold isopropanol, inverted to mix and stored at -20 °C overnight. After freezing, samples were centrifuged at 16,000 g at 4 °C for a total of one hour and the supernatant was pipetted off taking care to not lose the formed pellet. The pellet was then washed with 1,000 µl of 70% ethanol and centrifuged at 16,000 g for 10 min. Finally, the ethanol was pipetted off and pellets were allowed to air dry for 15 min, before re-suspending them in 100 µl TE buffer and

stored at 4 °C. DNA extracts from both extraction methods were tested for the presence of contaminants using a NanoDrop spectrophotometer.

Ant nest entrance soil samples were extracted using five methods: (1) the Biomeme M1 Bulk Sample Prep Kit for DNA – High Concentration (Philadelphia, Pennsylvania); (2) the CTAB method; (3) the PPLPP method (Edmunds and Burrows 2020); (4) the Qiagen Dneasy PowerSoil kit (Germantown, Maryland); and (5) the modular-universal DNA extraction (Mu-DNA) method (Sellers et al. 2018).

For the Biomeme M1 Bulk Sample Prep Kit for DNA – High Concentration (Philadelphia, Pennsylvania), we modified the previously-described procedure in the first and last steps: the equivalent of 1 ml of soil samples were initially transferred into 3 ml of Biomeme lysis buffer and DNA extracts were eluted in 400 μl Biomeme Elution buffer. Similarly, the first step of the CTAB method was modified, wherein each 1 ml-field replicate was split into two 2 ml DNA LoBind tubes (the equivalent of approximately 0.5 ml of soil sample/tube) containing 1000 μl CTAB buffer.

For the PPLPP method, each replicate of 1 ml soil sample was transferred into a 50 ml DNA LoBind Falcon tube containing 10 ml Longmire's buffer (Longmire and Baker 1997) and 10 ml of MilliQ water. Environmental DNA was extracted following Edmunds and Burrows (2020) with eDNA eluted in 100 μl elution buffer.

For the Qiagen Dneasy PowerSoil kit (Germantown, Maryland) (from hereon referred to as 'Qiagen method'), each field replicate consisting of the equivalent of 1 ml soil was partitioned into four tubes with the equivalent of 250 ml of soil and mixed with 60 μl Solution C1. The bead beating step was not performed given the target was not bacterial DNA from the soil samples. We then followed the manufacturer's protocol handling each field replicate in four separate tubes, sequentially passed through a single spin column and eluted in 100 μl elution buffer.

Finally, we followed the soil sample workflow of the Mu-DNA protocol without the bead beating step. Each equivalent of 1 ml soil replicate was split into two 2 ml DNA LoBind tubes and mixed with 550 μl lysis solution, 200 μl soil lysis additive and 20 μl proteinase K. Samples were then vortexed and incubated for 3 h at 55 °C. Subsequently, samples were centrifuged at 4,000 g for 1 min, the supernatant was transferred into a new tube, centrifuged at 10,000 g for 1 min and the supernatant was again transferred into a new tube containing 300 μl flocculant solution. Extraction was then carried out as published in Sellers et al. (2018). A negative extraction control was added to each batch of eDNA extractions to ensure that no contamination was introduced during laboratory procedures.

Quantitative real-time polymerase chain reaction (qPCR) and cycle sequencing

We screened samples for yellow crazy ant eDNA presence using two probe-based, species-specific eDNA assays developed and optimised by EcoDNA, targeting two separate sections of the yellow crazy ant Cytochrome Oxidase 1 (COI) gene region: Agra1 assay (112 base pair [bp] long) and Agra2 assay (131 bp long) (Suppl. material 1).

qPCR plates were set-up using the Arise Biotech EzMate 401 Automated Pipetting System (Taipei, Taiwan) and run in a Thermo Fisher Scientific QuantStudio 5 Real-Time PCR System (Singapore). We tested four technical replicates of each sample and

each site, including field and extraction blanks, three no-template controls and genomic DNA positive controls. Each qPCR reaction and cycling conditions were as explained in the assay development section of this study (Suppl. material 1). Inhibition was tested using a Thermo Fisher Scientific TaqMan Exogenous Internal Positive Constrol (IPC) qPCR assay (Burlington, Ontario). A total of 3 μl IPC was applied to duplicate samples and three reactions containing only IPC were included as controls. A departure of three or more cycles (ΔCt) would indicate sample inhibition (Hartman et al. 2005). Samples were subsequently purified using the Qiagen Dneasy PowerClean Pro Cleanup Kit (Germantown, Maryland) and another qPCR was carried out. Ct values between unpurified and purified samples were compared to assess the level of sample inhibition. A subset of amplicons with positive detections were Sanger-sequenced for confirmation of results at the Australian Genome Research Facility (AGRF). Resulting sequences were deposited in GenBank (accession numbers: MZ330820–MZ330832).

Data analysis

Differences in yellow crazy ant eDNA capture sensitivity (number of DNA copies per assay) across different methods were assessed with a generalised linear mixed model using a template model builder (TMB) computed in the R package glmmTMB version 1.7.19 (Brooks et al. 2021). The response variable was the DNA copy number and the explanatory variables were the eDNA extraction method (fixed effect) and field replicate/technical replicate (nested random effects, with technical replicates nested within field replicates). Two models were run: the first one testing replicates as an additive fixed factor and the second one testing replicates as an interaction. The best performing model was chosen, based on the corrected Akaike Information Criterion (AICc). We tested for overdispersion with the DHARMa R package version 0.4.4 (Hartig 2021). Post-hoc paired comparisons of means were performed using Tukey's HSD. Given that the differences in mean eDNA concentration across laboratory extraction methods were large, data were log-transformed solely for comparison (Fig. 3). Statistical analyses were completed in R statistical environment (R Development Core Team 2021).

Data accessibility

All data supporting the findings of this study can be found under the Suppl. materials and archived in the James Cook University Research Data Hub.

Results

Yellow crazy ant eDNA detection in water and soil

Water samples collected adjacent or in the vicinity of yellow crazy ant infestations showed positive eDNA amplification with both assays. The highest percentage of eDNA detections were observed at Stuart Creek (100% of field and technical rep-

licates using the Agra2 assay), followed by Ross River (80% and 60% of field and technical replicates using the Agra2 assay, respectively), Palmetum Creek (100% and 50% of field and technical replicates using the Agra2 assay, respectively) and Chauncy Crescent Creek (20% and 10% of field and technical replicates using the Agra2 assay, respectively) (Fig. 2). The Agra2 eDNA assay amplified DNA extracts from water samples at a higher percentage than the Agra1 assay, which failed to detect yellow crazy ant eDNA at Chauncy Crescent Creek (Fig. 2).

In soil samples, the Agra1 assay was more efficient at amplifying DNA extracts. Samples collected from ant transit sites showed high concentration of contaminants (Suppl. material 2: table S1) and complete inhibition was observed in eDNA samples tested using both eDNA assays (Suppl. material 2: table S2). After purification of DNA extracts, both assays successfully amplified 43–64% of the laboratory-extracted samples (Table 2, Suppl. material 2: table S3). However, *in-situ* extracted samples had a low percentage of positive detections (14–18%) (Table 2, Suppl. material 2: table S3).

There was a greater percentage of positive technical replications from nest entrance samples compared to transit samples for both assays and for field based and CTAB purification (Table 2, Suppl. material 2: table S4). In general, the Agra1 assay was more efficient at amplifying yellow crazy ant eDNA, reflected in higher eDNA yields across all extraction methods than the Agra 2 assay (Suppl. material 2: table S4). In addition, the CTAB, PPLPP and Qiagen extraction methods had the highest percentage of positive detections when using both assays (Suppl. material 2: table S4), whereas eDNA yield (number DNA copies/assay) was variable depending on the sampling site and eDNA assay used (Fig. 3, Suppl. material 2: table S4). Field and extraction controls from both the ant transit sites and nest entrance sampling events did not show positive amplification.

At Ross River, where we collected both water and soil samples, yellow crazy ant eDNA detections of both substrata were similar: 40–90% of positive soil field replicates (Agra1 assay) compared to 80% of positive water field replicates (Agra2 assay) and 28–78% of positive soil technical replicates (Agra1 assay) compared to 60% of positive water field replicates (Agra2 assay).

Comparison of eDNA yield across extraction methods

There were significant differences between mean number of DNA copies across all eDNA extraction methods at both sites and using both assays (Fig. 3). For Gieseman Road samples tested using Agra1 assay, the field-based method yielded significantly fewer DNA copies than the CTAB (F = -3.006, P = 0.0008), PPLPP (F = -3.888, P < 0.001) and Qiagen (F = -2.345, P = 0.0136) methods (Fig. 3). Additionally, the Mu-DNA method yielded significantly fewer DNA copies than the CTAB (F = 2.198, P = 0.0345) and PPLPP (F = -3.080, P = 0.0008) methods (Fig. 3). When using the Agra2 assay, we found that the CTAB method yielded significantly higher number of DNA copies than the field-based (F = -2.590, P = 0.0018), Mu-DNA (F = 4.295, P < 0.0001), PPLPP (F = 1.711, P = 0.0003) and Qiagen (F = 1.886, P = 0.0001) methods (Fig. 3). In addition, the Mu-DNA method had significantly higher number of DNA copies than the PPLPP (F = -2.583, P = 0.0001) and Qiagen (F = -2.409, P = 0.0004) methods (Fig. 3).
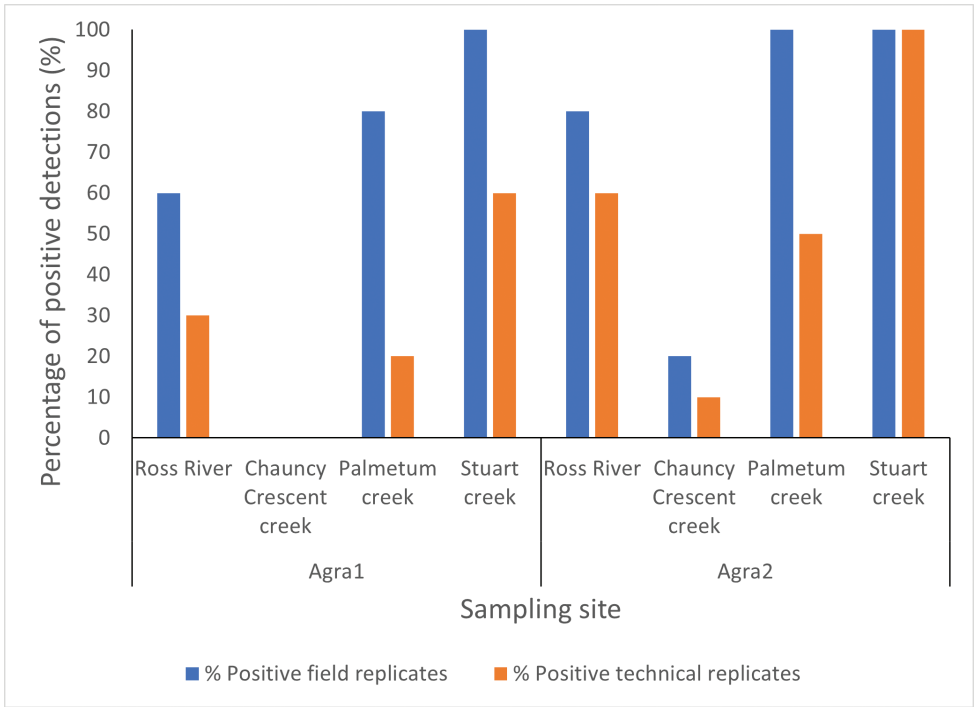
Figure 2. Percentage of positive yellow crazy ant eDNA detections from purified water samples using the Agra1 and Agra2 eDNA assays, targeting two different fragments of the COI gene.

For Douglas samples, the Qiagen method exhibited significantly higher number of DNA copies than the field-based (F = -7.477, P = 0.0002), CTAB (F = -4.399, P = 0.0115) and PPLPP (F = -6.051, P < 0.0001) methods of samples tested using the Agra1 assay (Fig. 3). When using the Agra2 assay, the field-based method yielded significantly fewer DNA copies than the PPLPP (F = -2.8819, P = 0.0036) and Qiagen (F = -3.5810, P = 0.0118) methods (Fig. 3); and the Mu-DNA method had significantly fewer DNA copies than the CTAB (F = 2.4420, P = 0.0077), PPLPP (F = -2.9663, P = 0.0039) and Qiagen (F = -3.6653, P = 0.0002) methods (Fig. 3).

Discussion

Detection methods that are sensitive to small number of individuals, such as eDNA analysis, have the capacity to complement and improve the detection of invasive species (Jerde et al. 2011; Smart et al. 2015; Villacorta-Rath et al. 2020). We used yellow crazy ants as a model species to test eDNA detection of a terrestrial invasive species in water and soil, as well as to explore the effect of soil eDNA extraction methods on eDNA detectability. Both substrata yielded positive yellow crazy ant eDNA detection. Importantly, we detected yellow crazy ant eDNA in water

Table 2. Yellow crazy ant eDNA detection in purified soil samples using Agra1 and Agra2 eDNA assays, targeting two different fragments of the COI gene. Extraction methods used were field-based (Biomeme M1 Bulk Sample Prep Kit for DNA – High Concentration [Philadelphia, Pennsylvania]), CTAB (cetyltrimethylammonium bromide, Adamkewicz and Harasewych 1996), PPLPP (preserve, precipitate, lyse, precipitate, purify method, Edmunds & Burrows, 2020), Qiagen (Qiagen DNeasy PowerSoil kit [Germantown, Maryland]), and Mu-DNA (modular-universal DNA extraction method, Sellers et al. 2018).

eDNA assay	Site	Extraction method	# Field replicates	% Positive field replicates	# Technical replicates	% Positive technical replicates
Soil samples – Transit sites sampling event						
Agra1	Gieseman Road	Field-based	7	29	28	18
		CTAB	7	86	28	64
	Copper Refinery	Field-based	7	29	28	14
		CTAB	7	71	28	50
Agra2	Gieseman Road	Field-based	7	4	28	14
		CTAB	7	57	28	43
	Copper Refinery	Field-based	7	29	28	14
		CTAB	7	57	28	43
Soil samples – Nest entrance sampling event						
Agra1	Gieseman Road	Field-based	10	70	40	45
		CTAB	10	100	40	90
		PPLPP	10	100	40	83
		Qiagen	10	80	40	78
		Mu-DNA	10	50	40	38
	Douglas	Field-based	10	40	40	28
		CTAB	10	80	40	68
		PPLPP	10	90	40	78
		Qiagen	10	70	40	48
		Mu-DNA	10	40	40	23
Agra2	Gieseman Road	Field-based	10	50	40	35
		CTAB	10	100	40	93
		PPLPP	10	100	40	100
		Qiagen	10	100	40	95
		Mu-DNA	10	70	40	58
	Douglas	Field-based	10	40	40	28
		CTAB	10	80	40	70
		PPLPP	10	70	40	70
		Qiagen	10	70	40	68
		Mu-DNA	10	50	40	35

samples from creeks and rivers directly adjacent and in the vicinity of known infestations. To the best of our knowledge, our findings are the first demonstration of the feasibility of detecting terrestrial invertebrate eDNA in natural waterways. Additionally, we found that eDNA detectability in soil is dependent on the extraction method and the area from which the samples are collected (i.e. ant transit areas vs. nest entrances) and that purification of DNA extracts is necessary to avoid false negative detections.
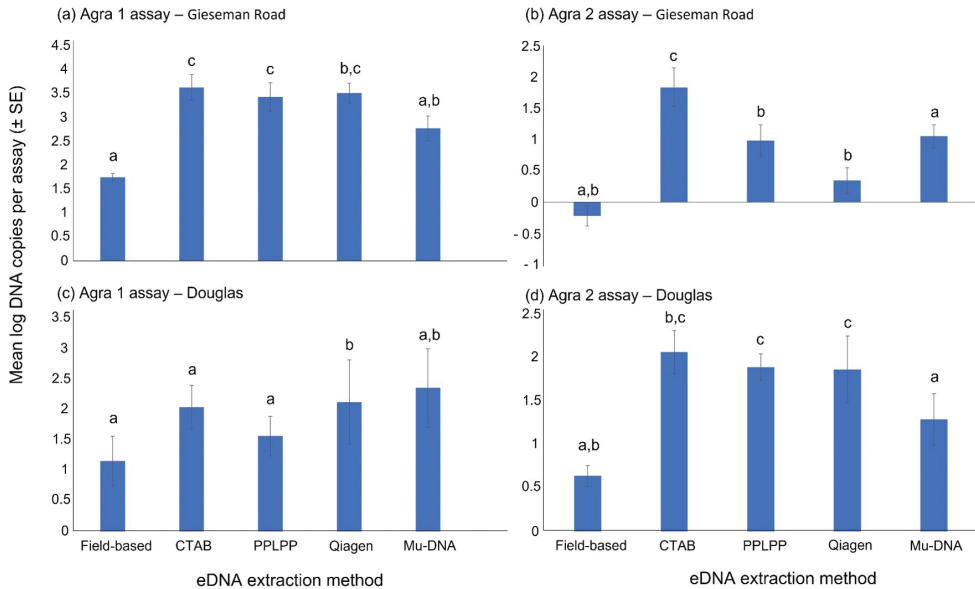
Figure 3. Mean DNA concentration (mean DNA copy number per assay ± Standard Error) yielded by each of the five eDNA extraction methods from purified soil samples collected from likely yellow crazy ant nest entrances (ant nest entrance sampling event) and run using the: (a) Agra1 assay on Gieseman Road samples, (b) Agra2 assay on Gieseman Road samples, (c) Agra1 assay on Douglas samples and (d) Agra2 assay on Douglas samples. DNA yield was $\log_{10}$ transformed. Note the differences in y-axis scales across panels. Methods with different letters above the bars within each panel differ significantly in post-hoc tests (at $P < 0.05$) using Tukey HSD. Extraction methods used were field-based (Biomeme M1 Bulk Sample Prep Kit for DNA – High Concentration [Philadelphia, Pennsylvania]), CTAB (cetyltrimethylammonium bromide, Adamkewicz and Harasewych (1996)), PPLPP (preserve, precipitate, lyse, precipitate, purify method, Edmunds and Burrows (2020)), Qiagen (Qiagen DNeasy PowerSoil kit [Germantown, Maryland]) and Mu-DNA (modular-universal DNA extraction method, Sellers et al. (2018)).

Many aquatic eDNA studies show that population size (Yates et al. 2019; Spear et al. 2021; Yates et al. 2021), time-elapsed since a target organism has occupied a system (Kucherenko et al. 2018; Schmidt et al. 2021) and target species behaviour (Buxton et al. 2017; Dunn et al. 2017), influence eDNA detectability in water. In the present study, qualitative data from the infestations adjacent to our water sampling sites suggested that ant activity (behaviour) was high in all sampled areas, except next to Palmetum Creek, where 80% of the field replicates exhibited yellow crazy ant eDNA. On the other hand, at Chauncy Crescent Creek, adjacent to an area of high ant activity, only 20% of the field replicates resulted in positive eDNA detections. Therefore, future research to disentangle the factors related to ant density and activity that could affect eDNA detectability in water will be useful. We also expect that the amount of rainfall prior to water sampling and the distance between the infestations and receiving waterbodies will play an important role in transporting terrestrial eDNA into the aquatic system. Once yellow crazy ant eDNA is in the aquatic system, we hypothesise

that the amount of rainfall will influence eDNA detectability by increasing water flow and dilution. As with aquatic eDNA studies, we would expect that the time elapsed since eDNA transport into aquatic systems would determine eDNA detectability due to factors affecting aquatic eDNA detection (i.e. eDNA production, decay, transport, retention and resuspension; see Barnes et al. (2016)). Future studies should focus on investigating four main factors that could influence yellow crazy ant eDNA detectability in waterbodies, namely: (1) total area of the infestation; (2) ant activity; (3) time since the establishment at a site; and (4) amount of rainfall prior to water sampling.

In soil samples, eDNA detectability from areas of yellow crazy ant transit was lower than that of ant nesting areas. A recent study on Argentine ants eDNA also found the highest eDNA concentration in soil from nest entrances, as opposed to surface soil samples from an infestation area and found no relationship between eDNA concentration and distance from nests or trails (Yasashimoto et al. 2021). The authors argued that Argentine ants may move nests frequently and, therefore, strong relationships between eDNA concentration and distance from a nest were not expected (Yasashimoto et al. 2021). Yellow crazy ants also move nests and transfer brood to different locations frequently (Lach pers. obs.) and, as with most ants, move dead ants to immediately outside of the nests (Hölldobler and Wilson 1990). Therefore, it is at the nest entrances where we would expect a significant amount of eDNA to be deposited. There is also the possibility that higher detections at nest entrance sites are due to small ant parts present in soil samples, even though we avoided sampling dead ants. If eDNA methods are used to check the progress of eradication efforts, this could constitute a source of false positive detections. Therefore, it would be important to investigate how to avoid the potential of false positive detections arising from dead yellow crazy ants. If the aim is to detect presence of the species in a new area, we propose the highest likelihood of collecting yellow crazy ant eDNA is in soil from samples at the base of trees or other moist areas where they are more likely to establish long-term nests. Yasashimoto et al. (2021) also concluded that the type of ant activity and their behaviour at different areas will determine eDNA detectability, indicating the importance of understanding the ecology of the species to avoid false negative detections.

Soil type may have also affected detectability. Samples collected from Gieseman Road, which has coarse sandy soils (Murtha 1975), showed a higher percentage of positive detections than those from Douglas, which has clay soils (Murtha 1975). Regardless of the eDNA assay used, soils with higher percentage of organic matter or clay and higher pH (i.e. more negatively charged) are more likely to bind to eDNA (Allemand et al. 1997; Andersen et al. 2012) and, therefore, inhibit the qPCR reaction. Therefore, we would expect to have more effective eDNA extraction from the coarser soil from Gieseman Road compared to the more organic-rich soil from Douglas (Murtha 1975), which is shown by the higher percentage of positive eDNA detections found at the former.

Our results showed that column-based eDNA extraction methods (Qiagen and Mu-DNA) perform better at removing sample inhibition than the other three methods, which only showed eDNA amplification after a purification step. This means

that the purification step could be avoided, cutting laboratory costs and shortening the sample processing time. In terms of eDNA yield, the Qiagen method was more or equally as effective in recovering eDNA from soil than the CTAB and PPLPP. Although the Mu-DNA method was less efficient than Qiagen, it can be scaled up to any starting volume of soil and it is almost ten times more cost-effective than the latter (Sellers et al. 2018).

Conclusions

In the present study, we used yellow crazy ants as a model species to explore eDNA detectability in two different substrata: water and soil. We demonstrated that terrestrial eDNA can be detected in waterbodies near yellow crazy ant infestations. Our findings suggest that there are opportunities for detecting terrestrial invertebrate eDNA across large areas given that mechanisms, such as rainfall runoff, could aggregate eDNA into nearby or downstream waterbodies. However, factors influencing terrestrial invertebrate eDNA detectability in water should be explored further. We showed that detectability of eDNA in soil is dependent on sampling location and the eDNA extraction method and that purification of DNA extracts is important to avoid false negative detections, making soil sampling less attractive than water sampling.

Acknowledgements

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Supplementary material 1

Invasive terrestrial invertebrate detection in water and soil using a targeted eDNA approach

Authors: Cecilia Villacorta-Rath, Lori Lach, Natalia Andrade-Rodriguez, Damien Burrows, Dianne Gleeson, Alejandro Trujillo-González

Data type: methods on assay development

Explanation note: This supplementary file contains the methods involved in yellow crazy ant eDNA assays development.

Link: https://doi.org/10.3897/neobiota.89.98898.suppl1

Supplementary material 2

Additional qPCR results

Authors: Cecilia Villacorta-Rath, Lori Lach, Natalia Andrade-Rodriguez, Damien Burrows, Dianne Gleeson, Alejandro Trujillo-González

Data type: Quantitative PCR results

Explanation note: This file contains additional data on qPCR results from eDNA samples.

Link: https://doi.org/10.3897/neobiota.89.98898.suppl2

RESEARCH ARTICLE

Intraguild predation as a potential explanation for the population decline of the threatened native fish, the European mudminnow (*Umbra krameri* Walbaum, 1792) by the invasive Amur sleeper (*Perccottus glenii* Dybowski, 1877)

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Abstract

Biotic interactions exerted by invasive species have a strong effect on ecosystems. Intraguild predation may contribute to the decline in the distribution, abundance and population size of native species and may facilitate the spread of non-native taxa. In this study, we investigated the feeding ecology of the invasive fish Amur sleeper (*Perccottus glenii* Dybowski, 1877) in a lowland watercourse, where it co-exists with the threatened native fish European mudminnow (*Umbra krameri* Walbaum, 1792). We used two sampling protocols that differed in the frequency of sampling time (e.g. monthly samplings and samplings in 10-day intervals) to provide evidence of predation, an interaction that may lead to the decline of mudminnow populations with the spread of the Amur sleeper. Aquatic macroinvertebrates comprised a major part of the diet for both sampling intervals. However, finer temporal resolution revealed the importance of fish, especially mudminnow juveniles, as a periodically available food source in the Amur sleeper's diet. A high degree of dietary overlap was found between the different size groups of the Amur sleeper, but larger

specimens tended to feed on a relatively higher proportion of fish. Our results suggested that temporal resolution of stomach content analyses may largely determine inferences on the importance of predation on juvenile mudminnow. Overall, we found that intraguild predation could contribute to the decline of European mudminnow populations, which underscores the importance of effective control measures to prevent the further spread of the invasive Amur sleeper.

Keywords
Diet analysis, endemic fish species, juveniles, population decline, trophic interactions

Introduction

Over the last few decades, anthropogenic activities and climate change have accelerated the spread of non-native freshwater fishes (Copp et al. 2005; Rahel and Olden 2008; Seebens et al. 2017) with devastating consequences on native biota (da Silva et al. 2010; Strayer 2010). Adverse trophic interactions between invaders and native species are usually exerted through competition and predation (Mooney and Cleland 2001; Foley et al. 2017). However, in species sharing trophic guilds, competition and predation can be combined, a phenomenon known as intraguild predation (abbreviated as IGP). IGP, which can be facilitated by phenology (e.g. spawning, the presence of juveniles) (Wissinger et al. 1996), increases the pressure on native fauna (Polis et al. 1989; Polis and Holt 1992) and modulates resource availability and, thus, the extent of competition versus predation (Taniguchi et al. 2002; Yurkowski et al. 2017). Besides, predator-prey interaction is defined by ontogeny (Werner et al. 1983) and by seasonal and spatial variability in species distribution (Winemiller 1990; Foley et al. 2017). Overall, the phenology of the predator and prey influence their roles as predator, competitor and prey (Gotelli 1995).

The Amur sleeper (*Percottus glenii* Dybowski, 1877) is one of the most intensively spreading invasive fish species in Eurasia (Copp et al. 2005; Reshetnikov and Ficetola 2011; Reshetnikov 2013; Horvatić et al. 2022). Originally found in the Far East of Russia, it is now on the "list of invasive alien species of Union concern (Union list)" within the scope of the new Regulation (EU) No. 1143/2014 on the prevention and management of the introduction and spread of invasive alien species (European Commision 2016). Due to the adverse impact of this invasive species on the recipient ecosystems, a thorough analysis of its ecological needs and invasion biology is necessary (Simberloff 2003; Reshetnikov and Ficetola 2011).

The Amur sleeper feeds on prey from several trophic levels (Reshetnikov 2003; Grabowska et al. 2009; Kati et al. 2015). It consumes mainly invertebrates (e.g. aquatic and terrestrial arthropods, molluscs and zooplankton), but fish can also form part of its diet when Amur sleepers are above 40 mm in body length (SL) (Sinelnikov 1976; Zaloznykh 1982; Grabowska et al. 2009). Consequently, the species potentially constitutes a serious threat to the native fish fauna, especially to the strictly protected European mudminnow (*Umbra krameri* Walbaum, 1792), which is an

endemic fish to the Danube and Dniester River Basins (Manteifel and Reshetnikov 2001; Witkowski and Grabowska 2012; Tatár et al. 2017; Grabowska et al. 2019). Species with a small distribution area (e.g. endemic species), short lifespan and low fecundity, such as the European mudminnow, are amongst the species most vulnerable to invasion (Clavero and García-Berthou 2005; Strayer 2010; Arthington et al. 2016). Similar to the Amur sleeper, the European mudminnow consumes primarily invertebrates, but rarely eats fish. It even participates in cannibalism in older ages (Lovassy 1927; Bănărescu 1964; Berinkey 1966; Wanzenböck 1995). Considering overlaps in feeding habits and habitats (Grabowska et al. 2019), IGP can be assumed between the two species.

The populations of European mudminnow strongly decreased after the river regulations of the Tisza River in the 19th century. Further declines accelerated after the establishment and spread of the Amur sleeper during the last few decades (Sallai 2005; Takács et al. 2015; Fazekas et al. 2016; Tatár et al. 2017; Bănăduc et al. 2022). Although the Amur sleeper is a superior competitor and reduces the foraging efficiency of the European mudminnow (Grabowska et al. 2019), declines in European mudminnow populations in the presence of the Amur sleeper were also noticed in habitats with unlimited food resources (Takács et al. 2015; Bănăduc et al. 2022). Accordingly, predator-prey interactions can be presumed between the Amur sleeper and the European mudminnow. Reduction of juvenile recruitment has been detected in several amphibian and fish species in the presence of the Amur sleeper (Spanovskaya et al. 1964; Litvinov and O'Gorman 1996; Reshetnikov and Manteifel 1997; Manteifel and Reshetnikov 2001; Reshetnikov 2001, 2008), but these observations require further, more detailed investigations in order to unequivocally prove the significance of the Amur sleeper in the decline of native fish populations.

To better understand the processes which may lead to the reduction of European mudminnow populations, we examined the role of predation in the biotic interactions between the invasive Amur sleeper and the native European mudminnow. Specifically, we examined the stomach contents of Amur sleepers in a lowland stream, where the mudminnow still has a dense population, but where the Amur sleeper has already established a population. Our objectives were as follows: (i) What is the importance of the mudminnow or other fishes to the food supply of the Amur sleeper? (ii) How does fish consumption depend on the size of the predator and prey? (iii) How does the temporal intensity of sampling influence the results of the diet analysis? We hypothesised that the Amur sleeper is an effective predator of mudminnow and it is especially effective on small young-of-the-year individuals since the Amur sleeper is a small-bodied predatory fish. Therefore, we also hypothesised that conventional seasonal or monthly stomach content analyses may be inefficient in quantifying patterns of fish consumption correctly. Specifically, we hypothesised that even monthly samplings are not effective enough to reveal the predatory effect of the Amur sleeper on the mudminnow since this may require more intensive samplings, which are better adjusted to the presence and growth rate of the mudminnow juveniles.

Material and methods

Sample collection and laboratory process

The sampling site was the lowland Hejő stream (coordinates: 47°52.0237'N, 21°0.1433'E) which is a tributary of the Tisza River, the second largest tributary of the Danube River. Our own fish surveys showed that the fish assemblage of the Hejő consisted of the following species: Prussian carp (*Carassius gibelio*) (2%), spined loach (*Cobitis elongatoides*) (3%), pike (*Esox lucius*) (2%), weather loach (*Misgurnus fossilis*) (3%), Amur sleeper (*Perccottus glenii*) (5%), roach (*Rutilus rutilus*) (4%) and European mudminnow (*Umbra krameri*) (81%). Consequently, the mudminnow still had the most abundant population at the examined site, while the Amur sleeper had the second-most abundant population. Note, that fish assemblage surveys in the Hungarian portion of the Tisza River Basin indicated a general decline of mudminnow populations with the spread of the invasive Amur sleeper (e.g. the extent of the mudminnow population decrease was over 95% in the Upper Tisza region) (Bănăduc et al. 2022).

The mean depth of the Hejő varies between 0.8 m and 2 m and the width averages 4 m. The stream is covered with dense aquatic and hydrophilic macrophytes (mainly *Lemna minor*, *L. trisulca*, *Ceratophyllum demersum*, *C. submersum*, *Hydrocharis morsus-ranae* and *Phragmites australis*).

Fish were collected using an electric fishing device (Hans Grassl EL64 II GI, DC, 300/600V max. 7 kW, Hans Grassl GmbH, Germany; permission number: HaGF/134/2019 and HaGF/68/2021) from March 2020 to August 2021. We designed two sampling protocols, which differed in frequency over time. First, we used traditional monthly samplings to characterise the diet of the Amur sleeper, similarly to other fish diet studies (Carman et al. 2006; Grabowska et al. 2009). Second, we applied a more intensive temporal sampling design (e.g. 10-day collections, see below) to test whether monthly samplings are representative for characterising the fish-eating behaviour of the Amur sleeper. During the monthly sampling, we collected 30 Amur sleepers in each month from March 2020 to February 2021 (samples were taken every 4 weeks starting from the 23 March (n = 360; collected specimens SL: 28 mm – 93 mm). To examine diet composition at a finer temporal scale, in a period when the Amur sleeper may prey on the eggs and larvae of potential prey (e.g. in the hatching and breeding season), we collected 20 specimens at 10-day intervals from the beginning of May 2021 till the end of August 2021 (n = 240; collected specimens' SL: 45 mm – 90 mm). Note: the Kolmogorov-Smirnov test did not indicate significant difference in the size frequency distribution of Amur sleepers between the monthly and the 10-day sampling protocols (D = 0.286; p = 0.304). In addition, mean length was also highly unlikely to be biologically significant, since it was 58.8 mm and 59.6 mm in the case of the monthly and 10-day samplings, respectively.

The collected specimens were euthanised by using clove oil in the field. The standard (SL) and total length (TL) were measured to the nearest 0.01 mm with a digital calliper. Sex and stomach fullness were determined by visual examination. Exenterated guts with their contents were preserved in 96% ethanol until dietary analyses. In the laboratory, gut

contents were determined under a stereomicroscope (EduBlue – ED.1802-S) and prey items were identified to the lowest practicable taxonomic level. The stomach fullness (in volume) was determined on a scale of 0–100% (empty – full) and the fullness contribution of each prey item category was estimated such that the sum of all prey categories equalled the total stomach fullness (Hyslop 1980; Amundsen et al. 1996; Kati et al. 2015).

Statistical analyses

Fish with empty stomachs were excluded from further analyses. To estimate the importance of fish, especially the European mudminnow in the diet of the Amur sleeper, we calculated the frequency of occurrence ($F_i\%$) and the percentage of prey-specific volume ($Pi\%$) for each prey category (Amundsen et al. 1996). The frequency of occurrence and the prey-specific volume were described by the following equations:

$$F_i\% = N_i \,/\, N \times 100$$

$$P_i\% = (\Sigma P_i \,/\, \Sigma P_{Ti})$$

where $F_i\%$ is the frequency of occurrence of the prey item i; N_i is the number of fish with prey item i in their stomach; and N is the number of fish with food content in their stomach. $P_i\%$ is the prey-specific volume of the food item i; ΣP_i is the stomach content (percentage) constituted by the prey item i; ΣP_{Ti} is the total stomach fullness of the fish, which contained the prey item i (Hyslop 1980; Labropoulou and Eleftheriou 1997). To investigate the food composition related to the body size of the predator, we divided the collected specimens into three size groups, based on the length-frequency distribution of the Amur sleeper population collected during the 10-day sampling protocol. The following size groups were established: small, ≤ 49 mm SL (n = 59); intermediate, 50–62 mm SL (n = 96); and large, ≥ 63 mm SL (n = 53).

We used non-metric multidimensional scaling (NMDS) with Bray–Curtis distance to evaluate the diet overlap amongst the size groups. NMDS is an indirect gradient analysis that generates an ordination, based on a specified number of dimensions and attempts to meet the conditions of a rank similarity matrix (Clarke 1993). The NMDS ordination is considered to be effective if the stress value (i.e. the measure of the match between distances in the original matrix and distances in the reduced ordination space) is below 0.2 (Clarke 1993). Diet categories that significantly (alpha = 0.05) influence the distribution pattern of the data points of the different size groups were determined using the "envfit" function (999 runs) in the freely available statistical programme "R" (version 4.2.2.) (R Core Team 2022). An analysis of similarity (ANOSIM) was also conducted to test the null hypothesis that there was no difference in the diet contents amongst the size groups. ANOSIM is a non-parametric test that compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. It produces a test statistic (R) expressed as a number between –1 and 1. An R value close to 1 suggests dissimilarity, while an R value close to 0 indicates similarity amongst groups. R values below 0 suggest that dissimi-

larities are greater within groups than between groups. The significance of the R statistic was determined by a permutation-based test (9999 runs) (Clarke 1993). Both NMDS and ANOSIM were run using the package "vegan 2.5.7" (Oksanen et al. 2020).

Dietary overlap between the Amur sleeper size groups was assessed using Schoener's (1970) dietary overlap index: $C_{xy} = 1 - 0.5\sum|p_{xi} - p_{yi}|$, where p_{xi} and p_{yi} are the proportions of prey i (based on the relative abundance of prey items) found in the diet of groups x and y, respectively. This index ranges from 0 (no diet overlap) to 1 (complete overlap). Schoener's index values > 0.6 are usually considered to be biologically meaningful in terms of consumed prey items by groups x and y (Wallace 1981; Clarke et al. 2005).

Results

Graphical analysis of the feeding strategy revealed that the Amur sleeper consumed mostly macroinvertebrates and had a broad dietary niche (Figs 1, 2). Although few food categories were consumed frequently with high prey-specific volume, the distribution of food items (points located at the upper left corner of the diagrams, see Figs 1, 2) indicated a substantial interindividual difference in diet content (resource partitioning).

In general, 15 and 12 prey categories were found, using the monthly and the 10-day protocol, respectively. Ephemeroptera and Crustacea dominated the diet, but Diptera and Coleoptera were also important in the monthly (Fig. 1) and the 10-day protocols (Fig. 2), respectively.

Diptera (March: 45%), Crustacea (May: 24%) and Ephemeroptera (*Cloeon dipterum* – April: 59%), as well as Odonata (*Coenagrion puella* – May: 23%) were the most abundant prey categories during spring in the case of the monthly protocol. The crustacean group (represented mainly by *Asellus aquaticus* and *Synurella ambulans*) was a major prey item by relative abundance in all seasons (June: 46%; November: 80%; January: 41%). Coleoptera (*Haliplus* sp.) was a main prey from summer (August: 17%) till winter (January: 17%), as indicated by its relative abundance, while Hirudinae, Heteroptera and Odonata were abundant mainly in summer (Hirudinae in August: 10%; Heteroptera in June: 6%; Odoniata in June: 14%) and autumn (Hirudinae in October: 15%; Heteroptera in September: 13%; Odonata in September: 16%).

During the 10-day protocol, the group Crustacea proved to be the most important prey (Fig. 2). Its contribution to the Amur sleeper's diet increased until the middle of summer (Fig. 2). Dragonfly larvae were a vital food resource from the end of spring till the middle of summer (Fig. 2). Amphibian tadpoles appeared continuously in the stomach from the end of June and became an essential forage base. The relative abundance of Ephemeroptera was remarkable at the end of spring (8 May: 66%; 18 May: 46%).

Piscivory was observed more frequently in the 10-day sampling protocol, with increasing importance from spring to summer. Fish eggs occasionally occurred in the diet in spring, but this diet category was amongst the less important diet categories (positioned low-right corner) (Fig. 2).

European mudminnow was the primary fish prey (Fig. 3); other fish species were identified in only two cases. Fish were usually consumed by only a few individuals

(moderate or low frequency of occurrence), but with substantial individual specialisa-
tion (high prey-specific volume). Specifically, we recorded the offspring of the spined
loach and the Amur sleeper in the stomach contents in one case.
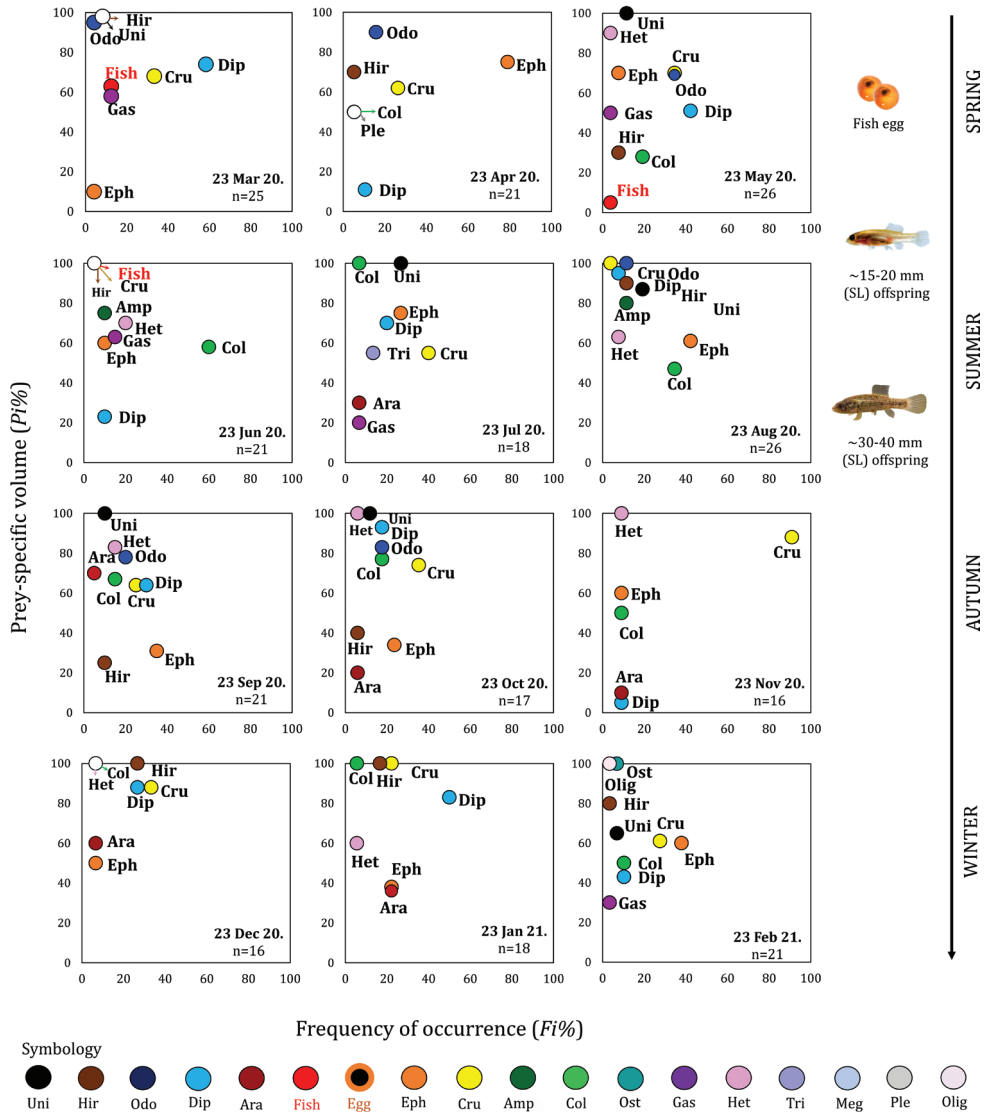
Figure 1. Graphical representation of the diet composition of Amur sleeper according to the method of
Amundsen et al. (1996) during the monthly interval sampling. Prey items identified as: Fish (fish); Amp
(Amphibia); Col (Coleoptera); Cru (Crustacea); Dip (Diptera); Eph (Ephemeroptera); Ost (Ostracoda);
Ple (Plecoptera); Olig (Oligochaeta); Ara (Arachnida); Gas (Gastropoda); Het (Heteroptera); Hir (Hir-
udinea); Meg (Megaloptera); Odo (Odonata); Tri (Trichoptera); Uni (unidentified food particles). Date
of the sampling occasions as well as the number (n) of the collected specimens (with non-empty stomach)
are presented in the lower-right corner. The diagram on the right side represents the temporal scale of the
sampling protocol and the ontogenetic development of the European mudminnow (0+).

Figure 2. Graphical representation of the diet composition of Amur sleeper according to the method of Amundsen et al. (1996) during the finer temporal (10-days) sampling. Prey items identified as: Fish (fish); Amp (Amphibia); Col (Coleoptera); Cru (Crustacea); Dip (Diptera); Eph (Ephemeroptera); Gas (Gastropoda); Het (Heteroptera); Hir (Hirudinea); Meg (Megaloptera); Odo (Odonata); Tri (Trichoptera); Uni (unidentified food particles). Date of the sampling occasions as well as the number (n) of the collected specimens (with non-empty stomach) are presented in the lower-right corner. The diagram on the right side represents the temporal scale of the sampling protocol and the ontogenetic development of the European mudminnow (0+).

Figure 3. European mudminnow (*Umbra krameri*) offspring within the digestive tract of an adult Amur sleeper (*Perccottus glenii*) female.

The NMDS analysis converged in two dimensions with a stress value of 0.13. The Amur sleeper size groups showed a high degree of dietary overlap (Fig. 4), resulting in a non-significant separation of groups (ANOSIM: R = -0.0063; p = 0.6062). However, larger specimens tended to feed on a relatively higher proportion of fish and had a greater niche breadth than specimens belonging to the intermediate and small size groups (Fig. 4).

Further quantification of diet overlap using the Schoener Index confirmed the results of the NMDS and ANOSIM analyses. The index values indicated high overlaps amongst the size groups, with the lowest similarity values between the small and large size groups (0.669), intermediate between the small and intermediate size groups (0.692) and the highest similarity between the intermediate and large size groups (0.865).
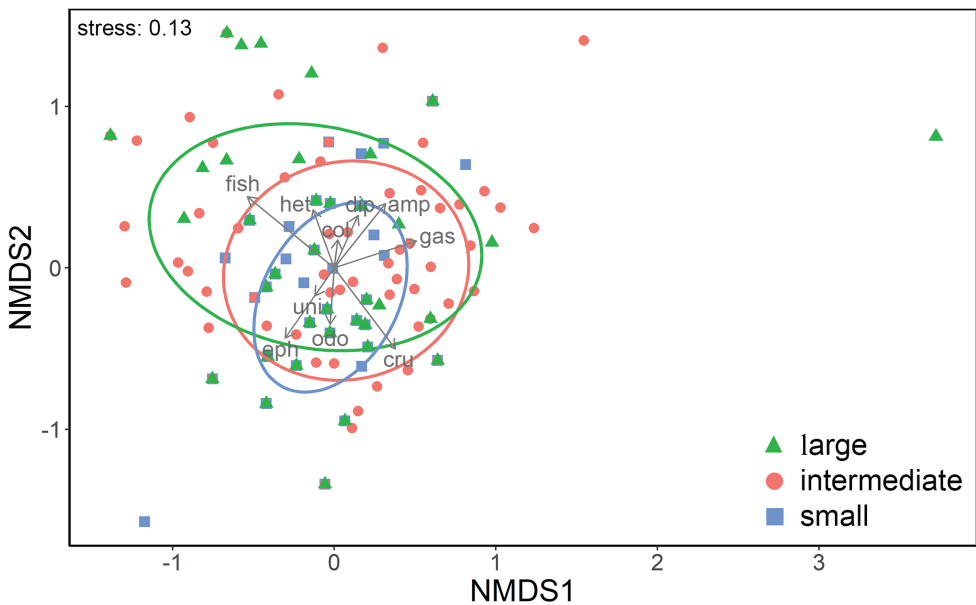
Figure 4. Non-metric multidimensional scaling (NMDS) analysis plots of Amur sleeper diet by size groups. Data points are coded and grouped by size groups. Ellipses illustrate the 95% confidence interval of the bivariate mean of a given size group calculated on standard error of the mean.

Discussion

Dietary analyses conducted at different temporal resolutions were useful to unambiguously prove the predatory effect of the invasive Amur sleeper on native taxa, especially on a small-bodied endemic fish like the European mudminnow. Conventional monthly samplings showed that the Amur sleeper is an effective predator of a variety of macroinvertebrates, but could not reveal the importance of fish in the diet. On the contrary, sampling at a finer temporal resolution highlighted that the Amur sleeper is feeding on fish eggs and is an effective predator of fish juveniles, which provides evi-

dence of predator-prey interaction. This interaction combined with competition for resources may be responsible for the decline of mudminnow populations in waterbodies that have been invaded by the Amur sleeper.

The Amur sleeper consumes predominantly macroinvertebrates (Koščo et al. 2008; Grabowska et al. 2009; Kati et al. 2015), which was also confirmed by our own samplings. However, fish consumption becomes more frequent with ontogeny (Koščo et al. 2008; Grabowska et al. 2019). As Amur sleeper is a non-selective, visual predator (Grabowska et al. 2009), motile fish larvae and tadpoles are more attractive (Reshetnikov 2008). Although we found that fish larvae and tadpoles were marginal food resources during the monthly samplings, the 10-day interval sampling highlighted the importance of fish eggs, juveniles and amphibians in the diet, especially in the hatching and breeding seasons. For example, during the monthly sampling, the frequency of occurrence ($Fi\%$) of fish in the stomach reached 12.5% (March) (Fig. 1), while during the 10-day method, this value reached 21% (egg - 7 June) and 33% (fish - 26 August) (Fig. 2). These values are remarkable compared, for example, with the results of Marsh and Douglas (1997), whose study examined the effect of predatory fishes on the endemic humpback chub (*Gila cypha*). Their results revealed that a lower frequency of prey item occurrence in the stomach (here 2% in the stomach of channel catfish (*Ictalurus punctatus*)) can indicate a considerable predation impact by non-native fishes, which can contribute to their population decline.

Amongst the consumed fish, the European mudminnow was the most dominant prey item. This is not surprising since this species was the most dominant fish in the community, which occupies the same meso- and microhabitats as the Amur sleeper (e.g. vegetated areas in the water column) (Pekárik et al. 2014; Grabowska et al. 2019). In addition, juveniles of the mudminnow grow more slowly than fingerlings of the Amur sleeper (Wanzenböck 1995; Nyeste et al. 2017). Consequently, the mudminnow juveniles are at risk of predation for a longer period than the juveniles of the Amur sleeper, especially in those populations where the Amur sleeper is gape-size limited due to the lack of large specimens in the population, such as we experienced in the Hejő. It is important to note that the narrow size range of the Amur sleeper may have influenced the results on the intensity of predation in the examined population since the predatory effect can be even stronger in those populations, where the size of the predatory fish is larger (Litvinov and O'Gorman 1996). Nevertheless, this relatively small size range is typical in most lowland streams in Hungary, where the Amur sleeper has dense populations (Nyeste et al. 2017).

IGP is determined by the predator and prey size; prey size range extends with the increasing body size of the predator (Scharf et al. 2000; Dörner and Wagner 2003). Indeed, fish consumption showed strong ontogenetic and seasonal patterns. Due to the gape-size limit, smaller Amur sleepers consumed fish sporadically (Grabowska et al. 2009; Kati et al. 2015), while the frequency of the occurrence and contribution of fish in the diet increased with increasing predator body size. High overlaps amongst the diet composition of the Amur sleeper size groups, especially between the intermediate and large size groups, were detected due to the narrow range of the body length of the

Amur sleeper population. Nevertheless, our results (i.e. Schoener Index values, NMDS and ANOSIM analyses) also suggest that the larger Amur sleepers had a greater dietary niche breadth and fed on a high portion of fish (Fig. 2), which can be even more explicit by the growing and ageing of the Amur sleeper population (Litvinov and O'Gorman 1996). Other studies found that the degree of dietary niche overlap can be smaller in populations that contain larger individuals that feed mainly on fish (Sinelnikov 1976; Zaloznykh 1982).

The seasonality patterns of IGP are driven by the availability of prey (Yurkowski et al. 2017) and IGP exerts pressure mostly on juveniles (Mehner et al. 1996; Fritts and Pearsons 2004; Hasegawa and Fukui 2021). Based on the finer temporal sampling protocol, juveniles of the European mudminnow were a periodically available resource. Feeding on fish eggs and mudminnow offspring were detected after the hatching season and consumption of juveniles increased during the survey. European mudminnow offspring over 25 mm in length has proved to be the most vulnerable ontogenetic developmental stage by the Amur sleeper predation. Fish consumption, instead of feeding on macroinvertebrates, can be more energy-consuming (Polačik et al. 2009); however, it is more valuable due to its high nutritional value (Elliott and Hurley 2000). In the following spring, one-year-old (0+) mudminnow specimens will grow over the gape size of the Amur sleeper and, thereby, the predation impact of the species decreases further. Note that, although cannibalism has been also found in the case of the Amur sleeper (Koščo et al. 2008; Interesova and Reshetnikova 2020), it was only observed once during the study.

The invasive Amur sleeper affects the food web of recipient ecosystems (Reshetnikov 2003) and threatens native species (Marsh and Douglas 1997; Reshetnikov 2008; Grabowska et al. 2009; Kati et al. 2015). Our results show that intraguild predation can play a role in the decline of the European mudminnow population by the elimination of mudminnow juveniles. The adverse effects of this multi-trophic interaction are more emphasised in species with low fecundity, short life spans and small distribution areas (Rocha et al. 2015). The absolute fecundity of the European mudminnow ranges between 100–2000 eggs/female and the maximal lifespan of the species is only 5 years (Kottelat and Freyhof 2007; Wilhelm 2008). Depending on the feeding conditions, the Amur sleeper can start feeding on fish in earlier life stages (Koščo et al. 2008) (when it reaches 45–50 mm body length in the 1st–2nd year (Nyeste et al. 2017)) and fish remains an important food resource afterwards (Sinelnikov 1976; Zaloznykh 1982; Litvinov and O'Gorman 1996; Grabowska et al. 2009; present study). This predation pressure on juveniles may lead to a collapse of European mudminnow populations within a few years after the establishment of Amur sleepers, as has previously been experienced in several cases (Bănăduc et al. 2022). Note that, although we could not investigate the direct predatory effect of mudminnow on Amur sleeper juveniles due to its conservation status (strictly protected, endemic species), large specimens of European mudminnow also consume fish (Lovassy 1927; Berinkey 1966; Wilhelm 2008). Therefore, a certain extent of predatory pressure on the Amur sleeper's offspring by the mudminnow cannot be ruled out in co-existing populations. However,

the longer life span (Nyeste et al. 2017), higher fecundity and aggressive behaviour of the Amur sleeper (Grabowska et al. 2011) increase the negative effects of IGP on the European mudminnow population. Since the distribution area of this endemic species is restricted mainly to the Carpathian Basin, further spread of the Amur sleeper may result in the extermination of the European mudminnow. Therefore, conservation measures (e.g. preventing further spread and the selective removal of the Amur sleeper) are more urgent than ever.

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RESEARCH ARTICLE

Aerial culling invasive alien deer with shotguns improves efficiency and welfare outcomes

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Abstract

Invasive alien deer (known in Australia as 'feral deer'; hereafter, 'alien deer') are some of Australia's worst emerging pest species. Recently, the Government of South Australia launched a four-year program to reduce the populations of alien fallow deer (*Dama dama*). The program will focus on coordinating landscape-scale aerial culls and seeks to deliver the most efficient and humane approach to aerial culling. We sourced data from a recent program trialling a new approach to aerial culling that incorporated advanced thermal technology and a second shooter with a shotgun to target fallow deer. We reviewed available video and audio records of 104 deer culled in the program to assess efficiency and welfare outcomes. We collected information on the number of shotgun and rifle rounds fired per animal, time between first shot with

a shotgun and apparent death, and pursuit time. We completed field dissections of 20 individuals targeted in the program to assess the lethality of wounds inflicted with shotgun pellets. We also compared program costs and efficiency against published and unpublished data from ten other aerial-culling programs for alien deer in South Australia since 2009. A total of 383 shotgun rounds and 10 rifle rounds were used on 104 fallow deer in the focal program. We documented strong improvements to animal welfare for alien deer targeted with shotguns. The mean (± standard error) time between first shot and apparent death with a shotgun was 11.1 ± 0.7 seconds; mean pursuit time between detection and apparent death was 49.5 ± 3.4 seconds. Pursuit time increased with subsequent deer controlled within a group; the maximum pursuit time for any individual was 159.0 seconds. All autopsied animals had received lethal wounds from shotgun pellets, with 100% receiving lung-penetrating damage and 70% also receiving heart-penetrating damage. While a program that uses a shotgun and rifle combined with a second shooter and thermographer can cost more to mobilise, the outcomes measured in cost deer^{-1} made it the most cost-effective approach of any program we assessed. Control options that deliver improved animal welfare outcomes and increase efficiency are desirable for managing expanding populations of alien deer in South Australia and elsewhere.

Introduction

Invasive alien deer (known in Australia as 'feral deer'; hereafter, 'alien deer') are some of Australia's worst emerging pests. The total number of deer in Australia increased from an estimated 200,000 in 2000 (Moriarty 2004) to around 2 million animals by 2021 (i.e., a ten-fold increase) (Government of South Australia 2022). Their impacts are now severe and include damage to native plants, competition with native animals, economic losses to primary industries (crops, pastures, horticulture, plantations) (Bradshaw et al. 2021), and human safety hazards from vehicle collisions. Alien deer are reservoirs and vectors of endemic animal diseases and have the potential to transmit exotic animal diseases, such as foot-and-mouth disease (Cripps et al. 2019). If left uncontrolled, within 30 years the economic impacts of alien deer are expected to cost Australia billions of dollars annually (BDO EconSearch 2022; Frontier Economics 2022).

Australia has six species of alien deer – fallow (*Dama dama*), red (*Cervus elaphus*), hog (*Axis porcinus*), chital (*A. axis*), rusa (*C. timorensis*), and sambar (*Rusa unicolor*); of all the alien deer species in the country, fallow deer are the most abundant and widespread (Centre for Invasive Species Solutions 2022b). They are also considered one of the most difficult deer species to shoot from a helicopter during aerial control programs, because they tend to hide in dense vegetation and run fast, darting quickly from side to side when being pursued (Hampton et al. 2022). These behaviours make accurate shots with a rifle difficult and can increase pursuit times and duration of suffering relative to other deer species (Sharp et al. 2022).

Adopting new technologies could enhance the efficiency of aerial programs and welfare outcomes for target animals. Recently, Pulsford et al. (2023) concluded that

thermal-assisted aerial culls were more effective than ground shooting when targeting sambar deer, and Cox et al. (2022) demonstrated improvements in both efficiency and welfare outcomes for fallow deer by incorporating thermal technology into their aerial programs. Government programs across Australia are trialling new combinations of firearms for different terrain and species of deer to improve the efficiency of culling operations. For example, programs have been trialling the use of shotguns to target alien fallow deer in New South Wales and the Australian Capital Territory (Hampton et al. 2022). While shotguns are routinely used by the New Zealand Government for aerial culling of alien deer (Forsyth et al. 2013) and in Australia for aerial culling of goats (*Capra hircus*) and pigs (*Sus scrofa*) (Sharp 2012a, b), they are not widely used for the control of alien deer in aerial culling programs in Australia.

Fallow deer are also the most abundant deer species in South Australia and the population is increasing despite the Government of South Australia supporting helicopter and ground-based shooting programs for more than 15 years. Recently, the State Government and Regional Landscape Boards launched a four-year program to reduce the populations of alien fallow deer in South Australia. The program focusses on coordinating landscape-scale aerial culls and aims to deliver the most efficient and humane approach to aerial culling. In that context, the State Government recently did a trial program (henceforth, 'P1') to test a new approach to aerial culling; it incorporated advanced thermal technology and a second shooter with a shotgun to target alien fallow deer.

Our study assessed the outcomes from P1 to examine the efficiency of the shotgun-rifle-thermal configuration compared to other configurations used in aerial culling programs delivered in the same region and across South Australia. We predicted that using the shotgun-rifle-thermal combination could: (*i*) improve animal welfare outcomes for target animals by minimising time between first shot with a shotgun and apparent death and pursuit time, and rapidly deliver fatal injuries to vital organs; and (*ii*) increase the efficiency and/or cost-effectiveness of the program compared to other programs delivered in the same region and across the State.

Methods

Program location and target species

The aerial culling trial program P1 occurred from 1–7 in October 2022, covering ~ 20,000 ha of private property in the Limestone Coast region of South Australia, about 300 km southeast of Adelaide (Fig. 1). The program targeted fallow deer – relatively small-bodied cervids with adult masses of 35–55 kg (females) and 50–97 kg (males) (West 2018). For comparison, sambar deer are Australia's largest deer and weigh around 230 kg (females) and 300 kg (males) (Centre for Invasive Species Solutions 2022a). We reasoned that the small size of fallow deer would increase the likelihood of shotgun pellets effectively penetrating the thorax compared to larger-bodied species.
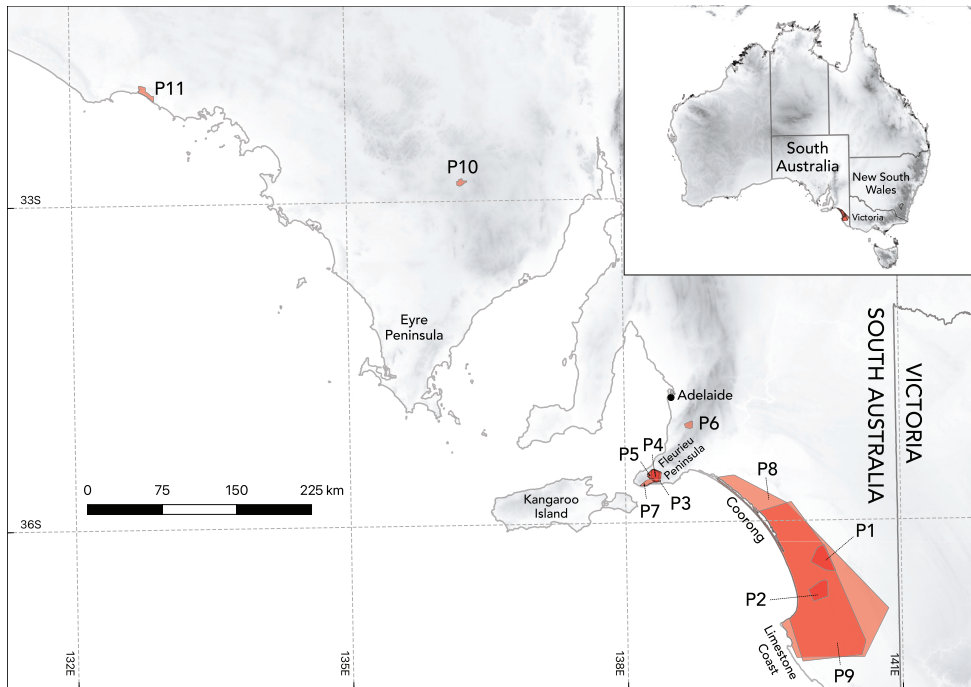
Figure 1. Location of the alien deer aerial culling programs in South Australia from 2009 to 2022 (P1–P11). See Table 1 for program descriptions. Red boxes are the minimum convex polygons enclosing all deer kills within each program (P1–P9), or the area searched by helicopters (P10–P11).

Firearms, ammunition, and crew configuration

All programs used either an AS350 B2 'Squirrel' (Airbus Helicopters, France) or Robinson R44 (Robinson Helicopter Company, U.S.A.) helicopter flown at altitudes generally below 250 m above sea level for all shooting operations. Shotguns can be used up to a maximum of 25 m from the target animal, so helicopters typically remained at 15–20 m above ground level at time of shooting. While rifles have a longer maximum range, shooters in the programs we describe do not typically take rifle shots at distances > 30 m from the target animal.

In P1, one shooter (hereafter, the 'primary' shooter) was equipped with a Benelli M2 semi-automatic shotgun with a 26" barrel and a custom choke at full extension, which created a 25-cm pellet spread at 20 m and a 45-cm spread at 30 m. The primary shooter targeted deer in open areas, within a 30-m range. The shotgun was fitted with a red-dot scope (Sightron S30-5 and Aimpoint 9000L); it had a 12-shell tube magazine and was loaded with GB SSG 21-pellet buckshot and Winchester Super-X 16-pellet buckshot. The projectiles of the 21-pellet SSG cartridges have an average weight of 1.8 g, with an average total payload of 37 g. The projectiles in the Winchester Super-X 16-pellet SSG cartridges have an average weight of 2.3 g and a total payload of 36 g. Professional shooters (Wildlife Resources Australia, Wangaratta, Victoria) did not observe any difference in the performance between the different rounds of buckshot.

Both round types were mixed into the primary shooter's ammunition bags, and we did not distinguish between ammunition type during data collection. The primary shooter was positioned in the rear right-hand side of the helicopter behind the pilot (Fig. 2), which gives that shooter the most-efficient position relative to the pilot manipulating the helicopter for optimal distance and angle relative to the target animal.

Another shooter ('secondary' shooter) was equipped with a Wedgetail WT25 semi-automatic, .308-calibre rifle with a variety of ammunition types. The ammunition included 160-grain copper projectiles used to cull deer near wetlands and creeks. Copper projectiles are being trialled in many pest-control programs in Australia because they do not contain any lead, but they could potentially increase the risk of ricochet (Steven Hess, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, National Wildlife Center, Colorado, personal communication). The secondary shooter targeted deer within vegetated areas and had a range of 70 m. The secondary shooter was positioned next to a thermal camera operator ('thermographer'; Fig. 2). The thermographer operated a Vayu HD uncooled microbolometer array with the Blackmagic Video Assist and Panasonic GH5 4K video camera and used a high-powered laser to assist the secondary shooter to locate deer in forested areas. The .308-calibre rifle was also equipped with a thermal scope (Pulsar Trail 2 LRF XQ50), so wounded deer in forested areas could be located quickly for follow-up shots and the thermographer could confirm death.
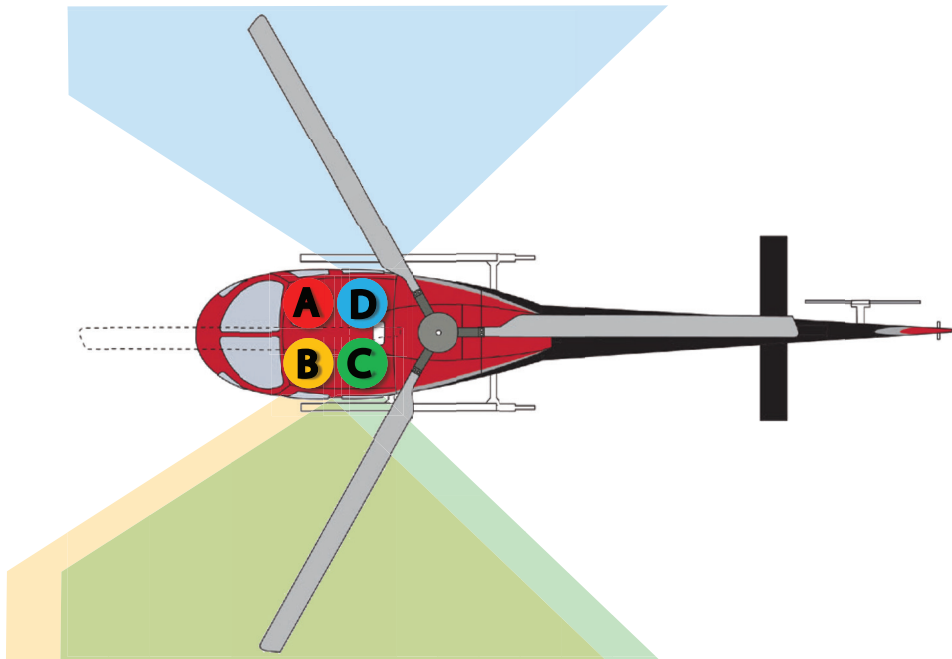
Figure 2. Seating configuration of the helicopter crew in P1 **A** pilot **B** secondary shooter with rifle and thermal scope **C** thermographer, and **D** primary shooter with shotgun and red-dot scope. Yellow and blue polygons show the indicative field of view for the shooters, and the green polygon shows the field of view for the thermographer.

Shooters made chest shots exclusively. For small deer species, especially those that move quickly and erratically such as fallow deer, chest shots are preferred for the best welfare outcomes (Sharp et al. 2022). P1 deployed a deliberate 'overkill' policy, which mandated that each deer was shot at least twice (following Hampton et al. 2022). If the target was not moving after a single shot, it would still receive at least one additional chest shot. Two crew members assessed both visually and with the thermal equipment the insensibility/death of each target animal before moving to the next target (see signs for assessing death in 'Data collection and analyses'). On average, the crew spent 5–10 seconds to determine each apparent death. The total flight time of P1 was 26.3 hours for a total of 611 alien deer culled.

All seating configurations and helicopter operational procedures are obliged to conform to the "Civil Aviation Safety Regulations 1998" and "Manual of Standards" produced and overseen by the Commonwealth of Australia's Civil Aviation Safety Authority (casa.gov.au). Safety therefore has primacy over all other considerations, including animal humaneness and efficiency/cost components of aerial shooting.

Data collection

All P1 flights were recorded on the thermal camera and with a GoPro 3 camera. The thermal camera captured all vision from the thermographer's perspective. The GoPro 3 camera was mounted to the rear firewall of the helicopter and recorded continuously; it captured the activities of all personnel in the helicopter and most of their field of view (Fig. 3). Both systems captured flight audio. The large video and audio files were overwritten every few days, so only a sub-sample of the 611 targeted deer was available for this assessment.

Figure 3. A GoPro 3 camera, mounted to the rear firewall of the helicopter, captured the seating configuration of the personnel in the helicopter, their field of view, and four deer being pursued (circled in red).

Based on the approach described by Cox et al. (2022), we reviewed all available video footage and audio from the first four hours of flight time on 2, 4, and 5 October 2022 and recorded: (*i*) number of shotgun and rifle rounds fired; (*ii*) time taken between the first shot fired at the target with a shotgun and apparent death (with shotgun or rifle); at least two helicopter personnel assessed time of apparent death based on the thermographer observing hotspots indicating that the thorax (heart and/or lungs) had been pierced, and a complete absence of movement determined by any crew member with clear vision; (*iii*) time between first detection of the target and confirmation of its death; if a deer stayed with its group under pursuit, pursuit time was cumulative for each consecutive deer (i.e., last deer killed in the group was recorded as pursued for the entire time that other deer in the group were being culled); if the group dispersed and a subset of that group had to be re-located, pursuit time was started when the group was relocated.

Analysis

To test which components of an individual kill explained the most variation in the time from the start of the pursuit to apparent death, we constructed a series of generalised linear models using the glm function in the stats R library (R Core Team 2022). Here, we tested whether the time between first and last/kill shots, number of rounds fired, and group size explained the variation in the time from the start of the pursuit to the kill (with a shotgun). We applied a gamma error distribution and a log link function to account for the non-Gaussian distribution of errors (confirmed appropriate after inspecting quantile-quantile plots), and scaled the response and explanatory variables (except group size) using the *scale* function in R. We contrasted a total of eight models, including the three additive main effects, all combinations of two additive effects, single effects, and the intercept-only model. We compared the relative probability of the five models per response variable using Akaike's information criterion corrected for small sample size (AIC$_c$) (Burnham and Anderson 2002). The bias-corrected relative weight of evidence for each model, given the data and the suite of candidate models considered, was the AIC$_c$ weight (the smaller the weight, the lower the model's probability) (Burnham and Anderson 2002). We also calculated the percent deviance explained (%DE) as a measure of goodness of fit. We examined model diagnostics using the *check_model* function in the *performance* R library (Lüdecke et al. 2021). All data and R code are available at https://github.com/cjabradshaw/deerCullShotgun.

Field dissections to assess lethality of shotgun damage

After the morning flights on 4 and 5 October 2022, 20 deer carcasses were located for assessment. Field dissections were done to collect information on shotgun-pellet penetration and spread and organ damage. Shotgun injuries were determined by cutting and peeling back the pelt and visually assessing the external muscle tissue for bruising and penetration of shotgun pellets on the impact and exit sides. Because damage from multiple projectiles to either the heart or lungs is lethal, the number of projectiles that impacted the thorax was also recorded for each carcass.

Following inspection of the muscle tissue and sites of pellet impact, the chest cavity was opened below the sternum using a bone saw. The heart and lungs were removed and inspected for tissue damage, wound channels, bleeding, and blood coagulation to determine whether pellets penetrated the heart and/or the lungs. The heart and lungs were dissected to establish the extent of the wounding by shotgun pellets, if not obvious externally. The chest cavity was also inspected for pooling of blood. All damage was recorded photographically, and the sites assessed for evidence of struggle or distress (such as kicking or disturbance of surrounding ground).

Cost-effectiveness

We compared the economic costs and outcomes of P1 to those of 10 other aerial culling programs (P2–P11) completed between June and November 2022. All programs targeted deer in the same region (Limestone Coast) or elsewhere in South Australia, and varied in crew configuration, firearms, equipment, deer density, area covered, and landscape (Table 1). P3, P4 and P5 were part of one large program, but we treated them separately based on their different configurations. We compared the programs according to the following metrics: (*i*) costs associated with delivering each program, (*ii*) costs per number of deer culled, and (*iii*) costs per flight hour and area covered.

Staff costs were included in the assessment because they are necessary to plan and deliver all aerial culling programs. This approach is consistent with 'competitive neutrality' requirements for government agencies in South Australia, which ensure government businesses compete fairly in the market (Government of South Australia 2023a). Staff costs were estimated to be $150 per hour for all agencies.

To contextualise any landscape-scale differences among the programs that could have affected cost effectiveness, we also calculated the dominant landcover classes within the area of each program using the South Australia Land Cover raster (2010–2015) at a resolution of 25 m × 25 m (available from data.sa.gov.au/data/dataset/sa-land-cover). We compared the land cover classes in which kills occurred to 'available' land cover classes within a minimum convex polygon defined by the locations of all kills in the program. Additionally, we calculated the mean human population density (persons km^{-2}) within 50 km of the program's minimum convex polygon to assess the relative likelihood of human visitors to a program area during culls (when near to larger human populations, personnel costs increase – see Results).

Results

Number of rounds

We reviewed all available footage from P1, which included 20% of the 611 fallow deer culled (*n* = 104). Of these, 92% were killed with a shotgun only (*n* = 96) and 8% with a shotgun-rifle combination (*n* = 8). Shooters used a total of 383 shotgun rounds and 10 rifle rounds (Table 2).

Table 1. Summary details of 11 alien deer aerial culling programs, including the recent trial (P1), to compare program efficiency. F = fallow deer (F); R = red deer (R); S = sambar deer (S); TAAC = thermal-assisted aerial cull (crew has a dedicated thermographer). All programs used .308 centrefire rifles exclusively except for P1 and P5 that also used a shotgun. The lead South Australian Government agency for each program was: PIRSA (P1–P5); Hills and Fleurieu Landscape Board (P6–P7); Limestone Coast Landscape Board (P8–P9); Eyre Peninsula Landscape Board (P10–P11).

No.	Region and location	Land use	Area (km²)	Deer species	Deer density	Helicopter	Primary shooter	Secondary shooter	TAAC	Shotgun	Notes
P1	Limestone Coast, Willalooka	rich agricultural area, isolated patches of vegetation	150	F, R, S	high	B2 Squirrel	✓	✓	✓	✓	current trial; fallow most common species
P2	Limestone Coast, Taratap	coastal agricultural area, linear vegetation remnant and dunes	100	F, R	high	B3 Squirrel	✓		✓		first trial of TAAC for deer in South Australia; fallow most common species
P3	Fleurieu Peninsula, Parawa	undulating peri-urban area mixed agricultural/rural with abundant	60	F	high	B2 Squirrel	✓		✓		Programs 3–5 delivered as part of a single program, but separated based on crew configuration, area covered, firearm type
P4	Fleurieu Peninsula, Parawa		30	F	high	B2 Squirrel	✓	✓	✓		
P5	Fleurieu Peninsula, Parawa	vegetated creek lines and vegetation pockets	110	F	high	B2 Squirrel	✓	✓	✓	✓	
P6	Adelaide Hills, Mt Bold	peri-urban water reservoir, undulating land covered in native and pine forest	20	F	high	R44	✓				goats also targeted
P7	Fleurieu Peninsula, Deep Creek	national park – undulating landscape with thick vegetation	40	F	high	B2 Squirrel	✓				
P8	Limestone Coast, Salt Creek to Taratap	coastal agricultural area, linear vegetation remnant and dunes	1200	F, R, S	high	2 × R44	✓				2 helicopters, single shooter in each; fallow most common species
P9	Limestone Coast, Salt Creek to Taratap	coastal agricultural area, linear vegetation remnant and dunes	1200	F, R, S	high	2 × R44	✓				2 helicopters, single shooter in each; fallow most common species
P10	Eyre Peninsula, Buckleboo	open, dry-land cropping country, isolated vegetation patches	160	R	low	R44	✓				no individual coordinates
P11	Eyre Peninsula, Chadinga	remote conservation reserve, squat coastal vegetation	100	no deer culled	low	R44	✓				no individual coordinates

Time between first shot with a shotgun and apparent death

The mean time between first shot with a shotgun and apparent death was 11.1 seconds (± 0.7; n = 104). Individual deer, or the first deer shot in a group, had the greatest mean time between first shot and apparent death, but this time decreased with subsequent individuals targeted within the group (Fig. 4). The maximum time recorded between first shot and apparent death for any individual deer was 35.9 seconds (Table 2).
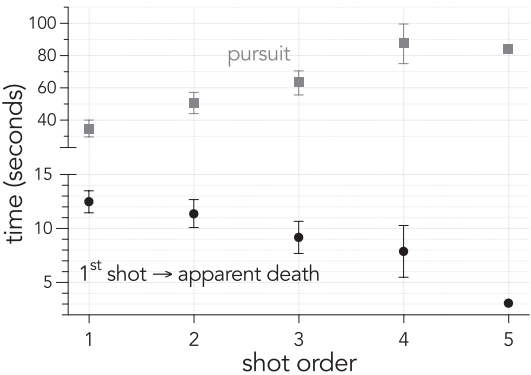
Pursuit time

Mean time between first detection and apparent death was 49.5 seconds (± 3.4; n = 104). Pursuit time increased with subsequent deer shot within a group (Fig. 4). The maximum pursuit time for any deer was 159.0 seconds. See summary data from the analysis of footage in Table 2.

Table 2. Summary statistics from footage of 104 deer killed with a combination of firearms, a secondary shooter, and thermal-imaging technology.

Summary statistic	Order of deer shot						
	First[a]	Second	Third	Fourth	Fifth[b]	Total	Mean
sample size (# deer)	45	29	21	8	1	104	–
shotgun rounds fired	169	114	64	34	2	383	–
mean ± s.e. shotgun rounds per deer	3.8 ± 0.3	3.9 ± 0.3	3.0 ± 0.4	4.3 ± 0.6	2.0	–	3.7 ± 0.2
rifle rounds fired	4	6	–	–	–	10	–
min-max time between first shot with shotgun and apparent death (seconds)	2.9–35.9	2.6–32.0	2.6–33.2	4.0–14.1	3.1	–	–
mean ± s.e. time between first shot with shotgun and apparent death (seconds)	12.5 ± 1.0	11.4 ± 1.3	9.2 ± 1.5	7.9 ± 2.4	3.1	–	11.1 ± 0.7
min-max pursuit time (seconds)	13.9–83.1	16.0–89.4	14.5–120.2	46.3–159.0	84.2	–	–
mean ± s.e. pursuit time (seconds)	34.9 ± 5.2	50.7 ± 6.5	63.1 ± 7.6	87.4 ± 12.3	84.2	–	49.5 ± 3.4

[a] first deer includes isolated individual deer as well as the first deer targeted within a group; data also collected for subsequent deer shot from the same group for up to five deer.
[b] sample size = 1, no standard error (s.e.), mean, or range calculated.

Figure 4. Mean (± standard error) time (seconds) between first shot and apparent death (black circles) and mean (± standard error) pursuit time (seconds) between first detection and apparent death (grey squares) as a function of shot order (either singularly or in groups of 1 to 5).

Model results

There was a positive effect of deer group size and number of shotgun rounds fired on the total time elapsed since start of pursuit to death (Table 3). These two variables explained ~ 43% of the variation in the response. However, there was no evidence for an effect of the time between the first and last shot and total time elapsed since start of pursuit to death.

Dissection to assess shotgun damage

The 20 carcasses were recovered and dissected within six hours of being culled in P1. All carcasses had received shotgun wounds only and were located using GPS data collected during the flight. A total of 116 shotgun pellets had penetrated the thorax of the 20 deer (5.8 ± 0.6 pellets deer^{-1}; range: 3–13 pellets deer^{-1}). Lethal lung-penetrating wounds were recorded in all 20 animals; 14 (70%) also recorded lethal heart-penetrating wounds. The wounds and their classification are shown in Suppl. material 1. Carcasses showed no indication of struggle or distress or movement from the location at which they were shot and apparent death by the helicopter crew.

Cost effectiveness

For 2022, the cost of delivering 11 aerial culling programs for alien deer in South Australia exceeded $1.1 million (Table 4); the mean ± s.e. cost per program was $100,461 ± $13,385; individual program costs ranged from $45,000 for one component of a larger program (P3) to over $160,000 for P8. As expected, the most expensive component of running any program was associated with helicopter operations, which comprised 54% of all costs.

Operating staff costs accrued by various agencies (South Australian Department of Primary Industries and Regions; Regional Landscape Boards of the Hills and Fleurieu, Limestone Coast, and Eyre Peninsula; National Parks and Wildlife Service;

Table 3. Generalised linear model results testing the effects of time between first and last/kill shots (*t1stLast*), number of rounds fired (*rnds*), and group size (*grpSize*) on the time from the start of the pursuit to the kill with a shotgun (response). k = number of model parameters; ℓ = -log likelihood; AIC_c = Akaike's information criterion corrected for small sample size; $wAIC_c$ ≈ model probability; %DE = percent deviance explained.

Model	k	ℓ	AIC_c	$wAIC_c$	%DE
~grpsize + rnds	3	-24.770	57.945	0.529	42.7
~t1stLast + grpSize + rnds	4	-23.859	58.330	0.436	43.7
~t1stLast + grpSize	3	-27.489	63.383	0.035	39.7
~grpSize	2	-32.480	71.201	0.001	33.8
~rnds	2	-50.879	107.997	<0.001	6.9
intercept-only	1	-54.745	113.610	<0.001	-
~t1stLast + rnds	3	-50.356	109.116	<0.001	7.8
~t1stLast	2	-54.603	115.446	<0.001	0.3

Table 4. Cost summary for 11 deer culling programs completed in South Australia between June and November 2022. P3, P4, and P5 are separate components of a large program; all staff hours were costed at $150 per hour. All costs in AU$ and include goods and services tax.

Detailed costs	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
helicopter operations	81,999	46,620	52,851	28,959	83,257	28,216	27,390	104,247	106,904	28,875	14,300
ammunition	7,500	1,868	2,802	1,535	4,413	2,756	2,200	4,051	3,221	0	0
professional shooters	7,200	3,000	3,842	2,105	6,053	6,916	4,500	27,000	27,000	4,200	1,780
PIRSA costs	20,625	26,149	18,010	9,869	28,371	6,450	0	0	0	0	0
Landscape board costs	5,625	970	1,890	701	2,659	29,100	21,375	16,950	9,000	31,000	6,750
NPWS costs	0	0	750	0	900	11,600	23,415	0	0	450	750
DEW costs	0	0	0	0	0	0	655	1172	1609	0	0
SA Water costs	0	0	0	0	0	47250	0	0	0	0	0
Forestry SA costs	0	0	2,500	1,500	3,000	0	0	0	0	0	0
community engagement	2,500	2,710	2,401	1,316	3,783	1,800	2,250	0	0	2,550	1,575
other logistics (car hire, travel, food, etc.)	4,700	2,460	2,145	1,175	3,379	1,900	2,600	6,846	6,978	3,100	2,200
Total costs	**$130,149**	**$83,777**	**$87,190**	**$47,160**	**$135,816**	**$135,988**	**$84,385**	**$160,266**	**$154,712**	**$70,175**	**$27,355**

Department for Environment and Water; SA Water; Forestry SA) varied considerably among programs. These costs were largely associated with the location of the operations. P3–P7 occurred on public lands (e.g., parks) near metropolitan areas, so additional staff were required to supervise entrances and prevent public access during the operations. Staff costs for all agencies for all programs combined exceeded $330,000, or 30% of all costs. P6 had the highest staff costs, exceeding $45,000, which comprised 54% of all costs associated with the project. This program required many multi-agency staff to supervise gates and entrances to the operations area, which is a high-profile, peri-urban site on public land (Fig. 1).

From the 11 programs, a total of 3,609 deer (at least 90% fallow deer) were culled during 486 flight hours (see Table 5). In terms of the program cost per deer controlled, P1 was the most cost-effective at $199 deer^{-1}. The least cost-competitive programs were P10 and P11, which operated in areas with low deer densities (Table 1). Seven animals were culled in P10, costing more than $10,000 deer^{-1}; P11 cost $27,000 and no animal was destroyed. Excluding P1, the cost per deer controlled in areas with high deer densities (P2–P9) ranged from $210 to $447 deer^{-1}. The cost per flight hour ranged from around $1,720 (P9) to $8,440 (P7); the mean was $4,526 ± $604 flight hour^{-1}; P1 cost around $4,950 flight hour^{-1}. The cost per area covered ranged from around $130 (P9) to $6,800 (P6) km^{-2} of program delivered; the mean was $1,445 ± $570 km^{-2}; P1 cost $868 km^{-2}.

Deer were most commonly killed in native woody vegetation > 1 m in height (64% of all kill locations across all programs) (Table 5), and in all programs except P7 (Suppl. material 1: fig. S6h), this land cover class was proportionally less-available (20% of area flown) (Suppl. material 1: fig. S6). Sparse native vegetation was the second-most common land cover class in which deer were killed overall (18%), which compares to an availability of only 1% (Suppl. material 1: fig. S6a). Dryland crops was the third-most common land cover class in which deer were killed overall (11%), but

Table 5. Cost effectiveness of 11 alien deer culling programs done in South Australia between June and November 2022. P3, P4, and P5 are separate components of a large program. All costs in AU$ and include goods and services tax.

Program outcomes	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
total animals culled	655	190	195	179	645	347[a]	243	645	503	7	0
total flight hours	26	16	18	10	29	20	10	87	90	21	9
animals/flight hour	25	12	11	18	22	17	24	7	6	< 1	0
cost/animal	$198.70	$440.93	$447.13	$263.46	$210.57	$391.90[d]	$347.26	$248.47	$307.58	$10,025	-
cost/flight hour	$4,948.63	$5,404.97	$4,777.58	$4,716.00	$4,724.05	$6,799.40	$8,438.50	$1,842.14	$1,719.02	$3,341.67	$3,073.60
cost/area (km²)	$867.66	$837.77	$1,453.17	$1,572.00	$1,234.69	$6,799.40	$2,109.63	$133.56	$128.93	$483.59	$273.55
dominant vegetation in program area	dry cropland	dry cropland	dry cropland	dry cropland	dry cropland	woody native > 1 m	woody native > 1 m	dry cropland	dry cropland	woody native[d] > 1 m	woody native[d] > 1 m
dominant vegetation in which deer were culled	dry cropland	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native > 1 m	woody native[c] > 1 m	
mean human pop density within 50 km (persons km⁻²)	0.47	0.52	69.70	75.76	68.64	58.05	59.50	4.71	3.96	0.10	negligible

a total animals culled for Program 6 at Mt Bold Shoot includes 61 goats
b the cost/animal adjusted to include the 61 goats is $333.30
c no individual kill locations available; value indicates dominant land cover class available (64% of area searched)
d no deer killed; value indicates dominant land cover class available (83% of area searched).

this was relatively low compared to an availability of 55% (Suppl. material 1: fig. S6a). Contrary to expectation, there was no apparent relationship between mean human population density within 50 km of a program and either the total personnel costs or personnel costs flight^{-1} hour^{-1} area^{-1} animal^{-1}; however, the Limestone Coast and Fleurieu Peninsula programs had separate clusters within this cost-population density relationship (Suppl. material 1: fig. S7).

Discussion

Aerial culling

Aerial culling can be an effective, rapid, and humane means for removing large numbers of alien deer (Husheer and Robertson 2005; Bengsen et al. 2022; Pulsford et al. 2023), alien pigs (Cox et al. 2022; Hamnett et al. 2023), and other pest species in vast, remote, and inaccessible landscapes. In 2020, 2021, and 2022, South Australia's aerial culling programs have removed approximately 3,000 alien deer per year (BDO Econ-Search 2022). In addition to aerial culling, some programs have used ground shooting by professional shooters, volunteers and landholders, and commercial harvesting operations (Government of South Australia 2023b). Recreational hunting and culling by private landholders are estimated to remove about 8,300 alien deer annually. With all control approaches combined, approximately 11,300 alien deer are removed per year from South Australia (BDO EconSearch 2022).

Unfortunately, a large proportion of the population of alien deer must be removed each year to drive population decline. For example, at least 34% of the population of fallow deer must be removed each year just to avoid population increase, and even higher culling proportions are required for other deer species (hog: 52%; chital: 49%; rusa: 46%; sambar: 40%) (Hone et al. 2010). The number of fallow deer removed annually from the estimated population of 40,000 in South Australia is around 28% (BDO EconSearch 2022), so the population has continued to grow.

Large-scale, intensive, and coordinated control programs are therefore necessary to drive population declines of alien deer. Improved efficacy of aerial culling programs is clearly needed if management goals to arrest the impacts of deer are to be realised. However, the adoption of new approaches and technologies first requires examination to ensure high animal welfare standards are met, in addition to operational cost effectiveness. Analysis of the outcomes from a recent trial program that used shotguns and thermal equipment, in combination with a rifle, provided insight into the humaneness and effectiveness of a new approach to controlling alien deer in South Australia.

Animal welfare

In pest control operations, welfare is generally evaluated in terms of the duration and intensity of suffering (Littin et al. 2004), which inform humaneness assessments of

control tools that are common practice in Australia (Sharp and Saunders 2011) and New Zealand (Littin et al. 2004). We used 'time between first shot with a shotgun and apparent death' and 'pursuit time' as indicators of duration of suffering and penetration and severity of shotgun pellets as indicators of intensity of suffering. The time recorded by Cox et al. (2022) between first shot and apparent death of deer using a rifle was 22 seconds; Hampton et al. (2022) reported that 95% of deer were dead within 57 seconds of the first shot in their program using rifles. In this trial, the average time between first shot with a shotgun and apparent death was 11 seconds, a markedly improved outcome for animal welfare.

Individual deer, or the first deer shot in a group, had the longest mean time between first shot and apparent death, and this interval decreased if targeting subsequent individuals in a group. This decrease is because of the relatively longer time taken to pursue a group of deer after first being sighted, before the first deer is shot. Once the group of deer was engaged, the pursuit time of the remaining deer in the group was usually shorter. The maximum time recorded between first shot and apparent death for any deer was 35.9 seconds, which is an improvement on programs that have used a rifle exclusively (Hampton et al. 2022).

Unlike Cox et al. (2022), our study assessed the metrics of a program that targeted deer with shotguns in relatively open terrain. Shotguns have not been trialled in densely vegetated areas, and so additional trials will be required to determine their efficacy in such habitats. Clearly, different vegetation densities and terrain will affect the outcomes of aerial culling program. The dominant vegetation class of several programs was 'dry cropland' (P1–P5, P8–P9), but only P1 also recorded this vegetation type as dominant where deer were killed. Unlike the other programs, outcomes from P1 included a subset of the overall program and selected for shotgun kills, which only occurred in open areas. We found similar proportions of available and kill-location land cover classes in P3–P4 (i.e., including P1, each had 50–60% dry cropland and deer were killed in 30–40% dry cropland; see S1), but the dominant land cover class where deer were killed for most programs was woody native vegetation (i.e., P2–P9) that harbour deer in the landscape.

Other influences such as proficiency of shooters, type of helicopter used, and weather conditions will also affect time between first shot (with shotgun or rifle) and death. In their study, Cox et al. (2022) measured the 'time from first shot impact to death', a potentially useful metric for assessing shooter proficiency. We were unable to differentiate impact shots from non-impact shots because the thermographer was not on the same side of the helicopter as the primary shooter with the shotgun. The GoPro footage was not of sufficient quality to assess individual shot impacts. However, we were able to assess overall pursuit time, and time between first shot and apparent death. Cox et al. (2022) and Hampton et al. (2022) recorded pursuit times of around 150 seconds and 90–200 seconds, respectively. The average pursuit time from 104 animals in our study was just 50 seconds, and the maximum pursuit time for any individual was 159 seconds.

In most jurisdictions, procedures and guidelines for aerial culling programs of alien deer dictate that a shot with a rifle is not taken until the shooter has a clear shot of the

chest or head, and that there is no risk of a wounded animal escaping to somewhere where a follow-up shot cannot be taken. The spread pattern of the shotgun pellets requires less precision for pellets to hit the thorax of the animal. Hence, using a shotgun reduces the time required to 'line up' an accurate and humane shot.

In terms of the intensity of suffering, all animals assessed had received rapid and lethal impacts from shotgun pellets. The average number of thorax-penetrating wounds delivered with the shotgun was higher than in some autopsies of deer culled with a rifle (Hampton et al. 2022). All animals recorded lethal damage to their lungs, and most to their hearts as well. Wounds to the lungs and the pooling and/or clotting of blood in the chest cavity indicated a pneumothorax (collapse of lung) and/or a hemothorax (collapse of lung because of blood in the chest cavity). The wounds to the heart are expected to have caused rapid decrease in blood pressure, rapid loss of consciousness, and rapid death by exsanguination. In combination, these injuries lead to hypovolaemic shock, causing unconsciousness due to inadequate cerebral perfusion pressure, and resultant rapid death from lack of blood supply to the brain (Stokke et al. 2018).

A potential shortcoming of our study is that the apparent death of the target animals in P1 was assessed in the air by the pilot, and at least one other crew member, rather than landing the helicopter to have a veterinary surgeon make a formal assessment (e.g., Hampton et al. 2022). Instead, a veterinary surgeon (A.D.) and a medical doctor (J.D.) were available for consultation for our study. Future research into the use of different firearms to cull deer could benefit from additional veterinary oversight, including work to ensure that culled deer do not have spinal injuries, which could render the animal unresponsive, but alert for some time. In addition, high-resolution photos taken from the helicopter could be used to compare the exact location and position of culled deer with photos subsequently taken from the ground. These records could be used to determine whether there were any signs of movement, distress, or disturbance of the surrounding ground after each deer was killed from the helicopter.

Cost effectiveness

Helicopter-based aerial shooting is a cost-effective tool for alien deer control (Bengsen et al. 2022). However, few studies have assessed the efficiency of different crew and equipment configurations. We assessed a trial program (P1) that used the same pilots, aircraft, and thermal technology as Cox et al. (2022) in their alien pig and deer control research. The main difference was the inclusion of a second shooter armed with a shotgun; it is only the second time (after P5) a program has used a shotgun for targeting alien deer in South Australia.

The largest expense associated with aerial culling is helicopter flight time (Bengsen et al. 2022), largely driven by the cost of aviation fuel. The approximate $2,500 cost hour[-1] of flight time for a B2/B3 Squirrel helicopter is nearly double that of the R44 (approximately $1,000). As such, when using the larger and more expensive helicopters in aerial culling of high-density deer populations, our results indicate that efficiency is maximised by the addition of a thermographer and second shooter with a

shotgun. While cost per flight hour and area is relatively high for P1, the efficiency of the configuration was unmatched (25 deer hour^{-1} at < \$200 deer^{-1}). Crew configurations would be amended to suit program objectives. For example, a second shooter or thermographer might not be necessary when targeting exclusively open areas where deer densities are high. However, the additional crew members reported other benefits, including (*i*) additional safety benefits because shooters had opportunities to take brief breaks during each flight; (*ii*) shooters had the opportunity to change roles when a magazine needed to be changed; (*iii*) shooters had the opportunity to alternate between using the shotgun and the rifle between flights; (*iv*) the thermographer had more opportunity to monitor welfare outcomes of targeted animals using the high-resolution thermal camera to confirm death and to locate wounded deer in forested areas; and (*v*) the thermographer provided a strategic approach to targeting alien deer and enabled searching and scanning areas harbouring deer that might otherwise be missed. The flight crew also reported an increase in the rate of detections of target animals because of the extra spotting capacity from an additional shooter equipped with thermal optics (Rob Matthews, Heli Surveys, Jindabyne, New South Wales, pers. comm.).

Program costs and efficiency will vary with location and density of deer. For example, the cost of targeting sambar deer at low densities in alpine environments exceeded \$1,000 deer^{-1} (Pulsford et al. 2023). We compared 11 aerial culling programs that varied in location, planning, staffing, and logistic requirements. P10 and P11 occurred in remotes areas with low deer densities. The goal of those programs was to eradicate small satellite populations before they established. The relatively high costs of programs in areas with low deer densities should not discourage land managers, particularly where eradication is possible. Of the programs delivered in areas with high deer densities, program costs ballooned for peri-urban programs because additional staff were required to restrict public access to popular recreation areas. Programs should continue to document the inputs, configurations, and outcomes of their efforts to inform future aerial culling programs of alien deer.

Conclusions

We found that the use of a suitable shotgun could improve welfare outcomes for culled deer, compared to programs that used .308-calibre rifles only. Improved welfare outcomes included reduced pursuit time and reduced time between the first shot and death. Furthermore, all deer dissected were shot more than once, and received multiple thorax-penetrating wounds, resulting in lethal injuries to either the lungs and/or heart, and ensuring a short time until death. These findings are at least as good as the best welfare outcomes reported from aerial culling programs in Australia to date (e.g., Hampton et al. 2022).

We found that a two-shooter crew configuration, with the addition of a thermal camera operator and a primary shooter with a shotgun, resulted in increased operational efficiency and cost effectiveness when compared to more conventional crew con-

figurations. These changes to the format of the aerial operation appeared to increase efficiency independently, but the addition of the shotgun appears to have made the biggest single difference. These results are likely to be applicable to areas with similar deer densities, canopy cover, and terrain to the Limestone Coast region of South Australia. Although thermal imagery can increase detection of control targets in denser vegetation, relative openness of the canopy will always be required for shooting to be efficient and effective. Control options that deliver improved animal welfare outcomes and increased efficiencies are urgently needed to manage expanding populations of alien deer in South Australia.

Acknowledgements

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Supplementary material 1

Supplementary information

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Data type: figures

Explanation note: Deer VI, killed with the shotgun; pelt is removed to show the difference between shotgun-pellet wounds on the entry (A) and exit (B) sides of the carcase. Deer XV, showing typical wounds and mode of death for feral fallow deer culled with shotguns in this trial. Deer IX, showing typical wounds and mode of death for feral fallow deer culled with shotgun in this trial. Deer XII, showing typical wounds and mode of death for feral fallow deer culled with shotgun in this trial. Deer XIV, showing typical wounds and mode of death for feral fallow deer culled with shotgun in this trial. Proportion of feral deer killed per major land cover class (black bars) relative to availability (proportional coverage within a minimum convex polygon defined by the kill locations per program; green bars) for (a) all kill locations combined and (b–j) P1–P9. Mean human population density within 50 km of the minimum convex polygon defined from kill locations per program (top panel).

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RESEARCH ARTICLE

Potential impacts of invasive crayfish on native benthic fish: shelter use and agonistic behaviour

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Abstract

Spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*) are successful North American invasive crayfish species distributed throughout Europe. Both species compete with native benthic fish for shelter. In a laboratory approach, we assessed competition for shelter and antagonistic interactions between these invasive crayfish species and the native benthic fish species, stone loach (*Barbatula barbatula*) and bullhead (*Cottus gobio*). This allows for studying the potential impacts of invasive crayfish on native benthic fish. Spinycheek crayfish and signal crayfish were able to gain control of the shelter and could successfully displace both benthic fish species. For stone loach, the presence of crayfish significantly decreased their shelter use and caused several behavioural changes such as reduced activity and increased hiding behaviour outside the shelter. Although the shelter use by bullheads was not reduced, they displayed similar behavioural changes, if less intense. Invasive crayfish species showed remarkable combative interactions against both species of benthic fishes, evidenced by the high number of aggressive interactions, especially concerning stone loach. Our results highlight the pronounced dominance of invasive crayfish over benthic fish in terms of shelter competition and aggressive interactions under laboratory conditions, which consequently might promote the latter's exposure to predation.

Keywords

Introduction

Introducing non-native species into a new habitat exhibits wide-ranging ecological impacts on native taxa, and thereby can globally threaten biodiversity to the entire ecosystem functioning (Carbonell et al. 2017; Flood et al. 2020; Mathers et al. 2020). Invaders are considered the second cause of biodiversity loss after the loss of habitat, particularly in freshwater ecosystems (Mack et al. 2000; Sala et al. 2000; Simon and Townsend 2003; Thomaz et al. 2015) because non-native species can displace native species, resulting in local extinctions (Vitousek et al. 1997; Mooney and Cleland 2001). Invasive species are responsible for the extinction of 34 species and partly responsible for 91 extinctions, causing severe changes in the structure and functioning of the invaded ecosystem (Parker et al. 1999; Clavero and García-Berthou 2005; Walther et al. 2009; Blackburn et al. 2011; Teixeira and Creed 2020).

Crayfish are among the most widely imported exotic freshwater taxa which are introduced to the environment by bait-bucket releases, intentional introduction to support fisheries, or release after educational use (Hobbs et al. 1989; Gherardi 2010). Non-native crayfish act as ecosystem engineers and represent a major threat to freshwater biodiversity, causing potential consequences across all levels of the ecosystem. Crayfish are omnivores, therefore they can affect both primary and secondary producers (Lodge et al. 2000; Perry et al. 2001). Population declines and extinctions of native species are among the most alarming outcomes of crayfish invasion (Lodge et al. 2000).

In direct contests between two competitors, morphological and physiological traits determining the potential to win these contests are defined as an individual's resource-holding potential (RHP) (Parker 1974). RHP of non-native crayfish as well as robustness in agonistic interactions restrict the access of native competitors to food resources (Gherardi and Cioni 2004; Larson and Magoulick 2009) and shelter (Gherardi and Cioni 2004; Nakata and Goshima 2006). Such exclusion from the use of limited resources can negatively impact the growth rates of native species (Pintor et al. 2008; Gherardi et al. 2011) and promote their vulnerability to predators (Garvey and Stein 1993; Mather and Stein 1993), thereby driving their decline (Capelli and Munjal 1982; Usio et al. 2001). Crayfish predation drives declines in diversity and abundances of native invertebrates (Mccarthy et al. 2006; Correia and Anastacio 2008; Mathers et al. 2018; Mathers et al. 2020) and reduces amphibian populations through direct predation on their eggs and larvae (Gamradt and Kats 1996; Gamradt et al. 1997). Accordingly, crayfish invasions resulted in fish declines through direct predation, shelter competition, and indirect competition for preys (Reynolds 2011). For several benthic species including crayfish, shelter availability is a critical factor for survival, allowing them to avoid predation by terrestrial mammals, birds, fish, or other crayfish species (Olsen 1973; Eggleston and Lipcius 1992; Hill and Lodge 1994; Lodge 1994; Englund 1999; Englund and Krupa 2000; Usio and Townsend 2000; Heinsohn et al. 2003; Almany 2004).

Two successful and important North American crayfish species invading Europe are the spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*).

Spinycheek crayfish was first introduced by the end of the nineteenth century, while signal crayfish was first introduced to Europe in the 1960s (Holdich 2002). Both species share some typical life-history traits of invaders such as fast growth, high fecundity, and early maturation (Souty-Grosset et al. 2006), which contribute to their invasion success. They also displayed a high reliance on shelters, potentially competing with the native benthic fish within European streams.

To better assess the potential consequences of crayfish invasion, this study was devoted to highlighting the competition for shelter between the invasive crayfish species, spinycheek crayfish and signal crayfish, and the native benthic fish species, *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead). Stone loach, bullhead, and crayfish species are similar in their ecological tolerances and habitat requirements. They are primarily nocturnal organisms that hide within shelters of rock crevices in streams and rivers (Miller 1984; Hill and Lodge 1994; Musil et al. 2010; Grabowska et al. 2016; Błońska et al. 2017). Due to the obvious spatial and temporal overlap in their shelter needs, it is anticipated that intense competition for shelter will occur, particularly during daylight hours. We, therefore, tested whether invasive crayfish would dominate benthic fish species concerning shelter use and if they express aggressive behaviour, thereby leading to behavioural changes in the benthic fish.

Materials and methods

Sampling and keeping

Crayfish and benthic fish specimens needed for laboratory behavioural studies were sampled between May 2018 and September 2019. We collected spinycheek crayfish from the right tributary (Nidda) in Schotten (50°28'N, 9°6'E) and the River Moselle near Koblenz, Germany (50°21'N, 7°36'E), while signal crayfish were sampled from the hyporhithral zone of the small river (Wied) near Neustadt (50°35'N, 7°26'E). All these sites are identified as invasion core areas. Stone loaches and bullheads were collected from a small gravel-bed Nister river (50°43'N, 7°44'E), where crayfish were observed but not abundant. All animals were active and used only once during the experimental procedures. Crayfish with missing or regenerating chelipeds or displayed moulting signs or had incomplete hardening were not considered for the experimental procedures. Crayfish were gathered using plastic rounded crayfish traps (60L × 26W × 24H cm) equipped with fish baits and dried dog food. These traps were set up along the shore overnight (ca. 0.5 m to 1 m depth). Benthic fish were collected by electrofishing (DK300, Brettschneider, Germany) with 350V DC and 4A. All animals were transported to the laboratory at Koblenz University. Crayfish were transported in a big dark container (66 L) lined with wet straw to avoid aggressive contact while benthic fish were transported in a 500 L container with aerated stream water. In the laboratory, 40 spinycheek crayfish and 40 signal crayfish were housed in plastic boxes (60 × 40 × 20 cm) in stocks, not more than three animals in one box (separated

by sex and species). Having benthic fishes collected one species at a time, 30 benthic fish were housed in two glass tanks (120 × 50 × 50 cm). All tanks and boxes were filled with dechlorinated tap water, contained a layer of gravel, and were equipped with PVC tube shelters (5 cm diameter and 15 cm length) more than the number of animals to avoid competition for shelter. All animals were kept under controlled conditions (light regime 16:8 L:D, water temperature 20.7 °C to 22.4 °C, pH 8.7–9.0). Benthic fish were fed frozen chironomid larvae whereas crayfish were fed on crabs' food JBL Novo Crabs tablets twice per week (food supplements with similar ingredients from other providers should be useful as well). Before the start of the experiments, all animals were acclimatised to lab conditions for seven days at least before starting the experiment. Body mass, standard length of benthic fishes, crayfish carapace length, and crayfish sex were recorded before each experiment (Table 1).

Behavioural experiments

In the shelter competition experiment, we performed 60 experiments with each benthic fish species (stone loach or bullhead), 20 trials with spinycheek crayfish and benthic fish species, and 20 trials with signal crayfish and benthic fish species. Furthermore, 20 control trials were performed with benthic fish only to observe their shelter use. For better observation, we built up 6 transparent glass aquaria (65 cm × 50 cm × 50 cm) with the same conditions in the acclimatisation tanks, providing only a single shelter in each trial (Fig. 1). All behaviours regarding the shelter and against the opponent were videotaped for 14h (6h during daytime, and 8h during night-time) using Raspberry Pi3 Model B. During the dark period, aquaria were lighted by infrared LED spots (Synergy 21, 10W SECURITY LINE, 850 nm). Each experimental trial started with introducing one species of benthic fish to the experimental arena and allowing them to acclimatise for 10h. Thereafter, one crayfish species was added to the experimental arena for 2h before starting the experiment to acclimatise.

For 14h, time spent inside the shelter by crayfish and benthic fish species was recorded during day and nighttime. Shelter occupation was considered when more than 50% of the animal's body was inside the shelter. Shelter status (occupied or not) was observed before the individual entered the shelter. If one species occupied the shelter and the other one entered the shelter, the reaction of the inhabitant (stayed inside, moved away, or evicted from the shelter) was recorded. The reaction of benthic fish (moving or not) to an approaching crayfish outside the shelter was also recorded. Meanwhile, the contest between the experimental species represented by crayfish aggressive movements and the retreating behaviour of the benthic fish species was also recorded. We noted: (1) the frequency of aggressive actions performed, (2) the type of aggressive and retreating behaviours observed which was then used to generate a behavioural intensity score (see Table 2), and (3) the duration of all contests during the observation period. Animals' behaviour was recorded every 30 min for 14h. An aggression score was assigned to each frame, using the score index from (Gherardi et al. 2013; Dalosto et al. 2015; Lopez et al. 2019) adapted to our species (Table 2).
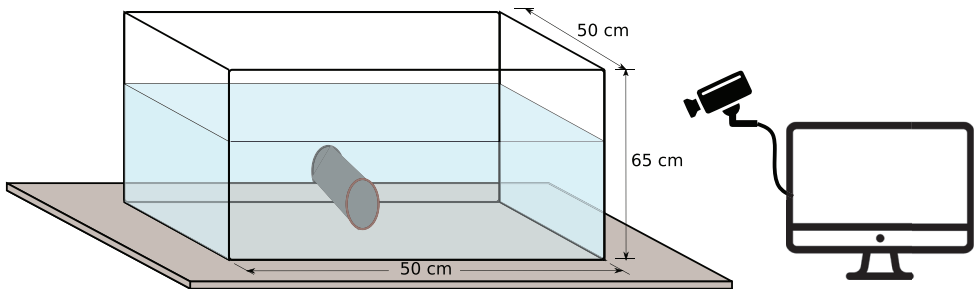
Figure 1. Model representing the experimental tank setup, showing the shelter position in the middle of the tank and the camera for recording the animal behaviour in front of the tank which is connected to a PC to observe and download the videos after recording.

Table 1. Mean (±SD) morphometric values of body mass and standard length (SL) of the native benthic fish stone loach (*Barbatula barbatula*) and bullhead (*Cottus gobio*) as well as body mass, carapace length (CL), and the number of males and females used in the experiment (*N* = 20 pairs) of the invasive crayfish species spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*).

Native benthic fish			Invasive crayfish				
Experiment	Body mass (g)	SL (cm)	Species	Body mass (g)	CL (cm)	Male	Female
Stone Control	2.40±0.59	5.4±0.56					
Stone loach + Spinycheek	3.10±1.10	5.6±0.58	Spinycheek	21.9±6.10	4.18±0.48	6	14
Stone loach + Signal	3.00±0.80	5.6±0.70	Signal	28.1±4.85	4.70±0.50	7	13
Bullhead Control	5.25±2.33	5.5±0.90					
Bullhead + Spinycheek	5.75±1.94	6.0±0.65	Spinycheek	29.4±7.96	4.60±0.61	11	9
Bullhead + Signal	7.00±2.70	6.3±0.94	Signal	32.0±6.60	4.70±0.46	14	6

Table 2. Ethogram of the behaviours observed in this study and the associated intensity score based on previous assessments of crayfish contests (Gherardi et al. 2013; Dalosto et al. 2015).

Behavior		Description	Score
Non-aggressive interactions		An approach without any agonistic reactions	0
Aggressive interactions	Antenna wave	The antennae of crayfish are whipped rapidly over the opponent.	+1
	Cheliped touching	Aggression with closed chelae: touching and pushing the opponent.	+2
	Cheliped half raise	The spreading and half raising of the chelipeds while facing an opponent.	+3
	Cheliped full raise	The spreading and full raising of the chelipeds while facing an opponent.	+4
	Grappling and pull	Intense combat: animals performing several agonistic acts simultaneously, trying to grab and pull the opponent's body. Kill the opponent.	+5

Data analysis

During video analysis, different behaviours were noted: (1) shelter occupation (measured as time spent inside the shelter), (2) shelter status (occupied or empty), and (3) agonistic behaviour. To do so, we developed a multi-object tracking algorithm to track the movement of the fish and the crayfish inside the experimental tank in the recorded videos. This algorithm was inspired by the procedures used by (Amen et al. 2020). The

basic idea of the algorithm was to compare each frame of the video with a background image, where no moving objects exist, to identify the pixels which undergo a colour change. Those pixels describe the exact location of the moving objects in the respective frame. The video-tracking algorithm was coded in Matlab (R2018b, MathWorks Inc., Natick, MA, USA). This routine was consistently validated for each video by comparing the actual locations with the predicted locations of the fish and the crayfish for randomly selected frames (1000 frames) to confirm its accuracy. For each video, we calculated a hit-rate value based on the following equation:

$$q = \frac{100}{n} \sum_{i=1}^{n} Ni \tag{1}$$

where q is the hit-rate in percent, n is the number of frames (1000 frames), and N is the hit value (1 for a correct prediction and 0 for an incorrect one). We accepted the automated process only for q values higher than 95%. Otherwise, we manually set the fish and the crayfish locations in each frame of the videos which scored q values less than 95%. We further processed the outputs of this routine, i.e., the generated spatio-temporal database of the moving objects (fish and crayfish), to observe the shelter occupation time, covered distance, and activity of the fish and the crayfish inside the experimental tank. For visualization, a spatial heatmap was used to show how the species locations are clustered or vary over space in the experimental tank. The colour variation represents the intensity of species locations in a 2-D form. Heatmaps were used also to measure the habitat proximity relative to the shelter, as a focal patch, to infer the potential for animal movement close to the shelter. The index of habitat proximity, H_x, is calculated, following (Winfree et al. 2005), using the weighted mean patch area for all the patches in the heatmap as follows:

$$H_x = \frac{\sum_{i=1}^{n} A_i e^{-d_i/D}}{\sum_{i=1}^{n} e^{-d_i/D}} \tag{2}$$

where A_i is the area of patch i, d_i is the distance between the shelter and patch i considered overall patches in the system, and D is a mobility constant scaled to the fish (diagonal of the tank). High H_x values indicate the high proximity of a fish to the shelter and vice versa.

The activity of the fish is calculated, following (MacLean et al. 1982), using the activity index (AI) to characterize the covered distance and speed of the fish. The maximum expected distance (d_m) and speed (s_m) were used to scale the distance and speed of the fish. We rewrote the original AI equation suggested by (MacLean et al. 1982) in the integral form so that it matches the spatiotemporal database. Therefore, AI was calculated as follows:

$$AI = \int_{t=t_1}^{t=t_2}(d/d_m)(s/s_m)^{0.5}dt \tag{3}$$

where t_1 and t_2 are the starting and ending times of the required period of fish activity. To calculate all these quantities, we used a Matlab post-processing script, which was designed specifically for this study.

To assess the impact of crayfish species on benthic fish species, we compared shelter occupation time as an independent variable between experimental groups using R software (R 4.2.1). In this study, shelter occupation time was non-normal distributed, over-dispersed (variance of the distribution greater than mean), and contained excessive zeroes. Consequently, a zero-inflated regression model (with Poisson errors and negative binomial errors) was performed by applying the "zeroinfl" function in the "pscl" package (Jackman 2012). To assess the difference in shelter status (occupied or unoccupied) between benthic fish and crayfish species prior to entering the shelter, we employed the McNemar Test.

To determine which benthic fish species faced more aggressive behaviour from the two crayfish species, and which crayfish species exhibited the most aggressive actions towards benthic fish, we transformed our non-normal distributed data in terms of aggressive movement scores and durations. Data from spinycheek crayfish and signal crayfish towards stone loach and bullhead were transformed using arcsine-square root. Afterwards, two-way ANOVA was performed to compare the score and the duration of aggressive movements in the different groups. Accordingly, two-way ANOVA was used to evaluate the influence of crayfish sexes on the score of aggressive movements towards stone loach and bullhead. Since the transformation to achieve the assumptions of normal distribution and homogeneity of variances was not possible, the Mann-Whitney test was used to compare the frequency of aggressive acts from both crayfish species (Table 2). In addition, a spearman correlation was computed to determine the relationship between the frequency of aggressive movements, crayfish carapace length, chela length, crayfish body mass, benthic fish body mass, and benthic fish standard length in each benthic fish group. These statistical analyses were performed in SPSS version 26.

Results

### Shelter occupation and habitat use

The presence of invasive crayfish species significantly affected the shelter occupation time of both benthic fish species. Stone loaches reduced the time spent within the shelter in response to both crayfish species. This reduction was more intense in the case of spinycheek crayfish during the daytime, resulting in a significant interaction term (Table 3, Fig. 2a, b). In the case of bullheads, the time spent in the shelter was
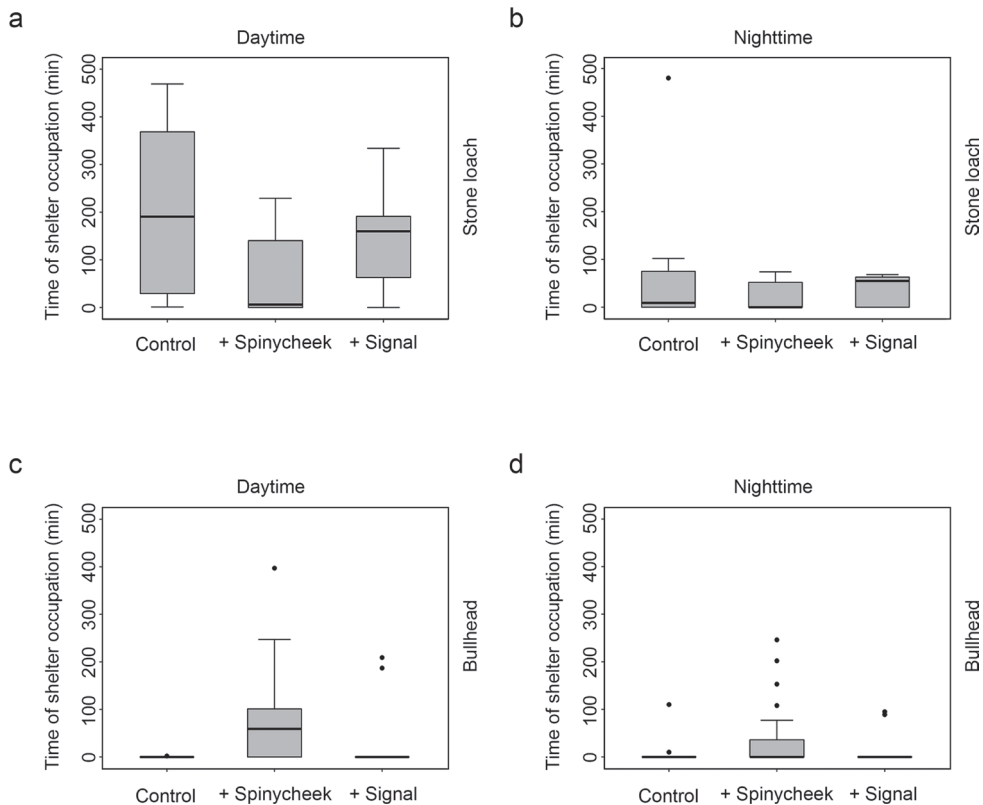
Figure 2. Box plots of shelter occupation time of the benthic fish species stone loach (**a, b**) and bullhead (**c, d**), kept either alone (Control; *n* = 20) or in the presence of spinycheek crayfish (+Spinyceek; n = 20) or signal crayfish (+Signal; *n* = 20) during the day and night times (line: median, box: 25,75% percentiles, whiskers: 5%,95% percentile, dots: outliers).

significantly prolonged, but only in the presence of spinycheek crayfish (Table 3, Fig. 2c, d). The entrance into an already occupied shelter was observed more often for crayfish (20 up to 40 times, Fig. 3) than for benthic fish (3.6 up to 17.2 times), as demonstrated by the significant difference in the proportion of shelter status before occupation between crayfish species and benthic fish species (p = 0.001, McNemar test). Notably, both benthic fish and crayfish shared the shelter just once (1× stone loach with spinycheek crayfish, 1× bullhead with spinycheek crayfish). On both occasions, the benthic fish escaped quickly from the shelter. Both benthic fish species were more often evicted from the shelter by signal crayfish (stone loach: 62%, bullhead: 55%) than by spinycheek crayfish (stone loach: 37%, bullhead: 45%).

Both crayfish species spent more time inside the shelter during the daytime than the nighttime. Therefore, the stone loach stayed for a long time, more or less stationary, in certain spots outside the shelter. This behaviour increased in the presence of both crayfish species during day and night (Table 4, Fig. 4a). Likewise, bullheads settled
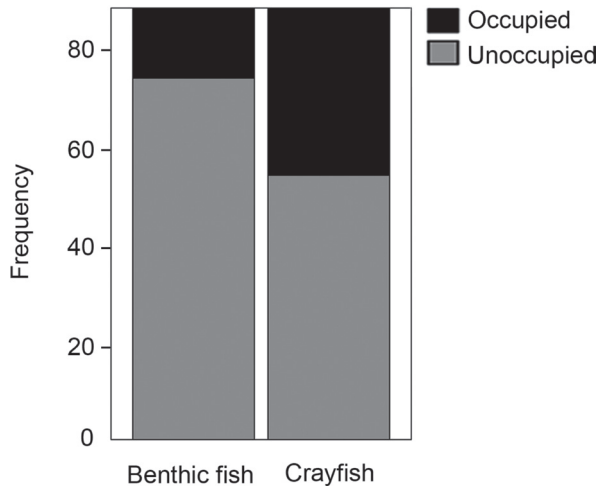
Figure 3. Status of a shelter (occupied: Black, unoccupied: Grey) before entered by crayfish or benthic fish.

Table 3. Results for the ANOVA's (generalized linear models) regarding the effect of the two crayfish species (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish) on the shelter occupation time of the benthic fish species *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead), during night and day, given as an estimate, standard error (SE), z-value, and *p*-value. Due to the different distributions, a zero-inflated distribution was used for bullhead and a Poisson distribution for a stone loach. Significant results are indicated by bold font.

Fish	Factor	Est.	SE	z	*p*
Stone loch	Intercept	5.31	0.015	338.3	< 0.001
	Signal	-0.22	0.025	-8.8	**< 0.001**
	Spinycheek	-0.37	0.030	-12.5	**< 0.001**
	Time	-1.01	0.035	-29.1	**< 0.001**
	Signal: time	-0.06	0.056	-1.1	0.261
	Spinycheek: time	0.13	0.060	2.3	**0.020**
Bullhead	Intercept	2.70	1.113	2.4	0.015
	Signal	-0.51	1.339	-0.4	0.705
	Spinycheek	-3.11	1.203	-2.6	**0.010**
	Time	-0.51	1.339	-0.4	0.705
	Signal: time	0.51	1.705	2.3	0.766
	Spinycheek: time	1.32	1.487	0.9	0.376

in a stationary position in particular spots inside the tanks for long periods (Table 4, Fig. 5a). Both benthic fish species changed their behaviour and were on average further away from the shelter when crayfish were present, which is indicated by a reduced habitat proximity index H_x (stone loach control: 0.52 ± 0.02, with spinycheek crayfish: 0.07 ± 0.003, with signal crayfish: 0.16 ± 0.01; bullheads control: 0.46 ± 0.05, with spinycheek crayfish: 0.33 ± 0.01, with signal crayfish: 0.26 ± 0.01). Similar to benthic fish, both crayfish showed stationary time inside the aquaria (Table 5, Figs 4, 5).
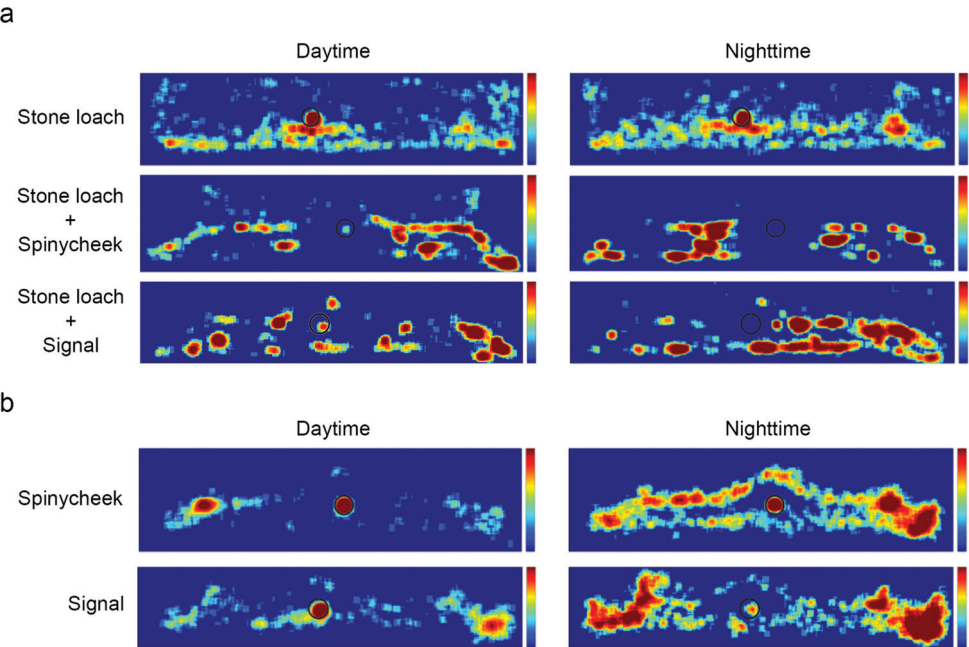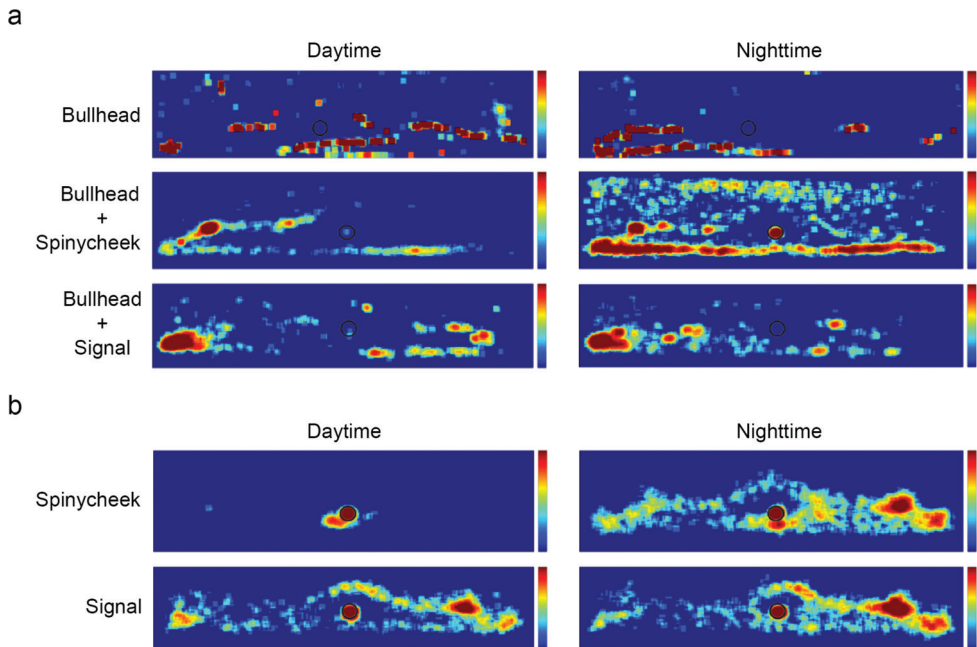
Figure 4. Heatmaps (shifting from blue over yellow to red with the increase of time spent in this respective location) is a graphical representation of the shelter occupation and animal distribution in the experimental tank **a** heatmaps showing the movement of (stone loach, *Barbatula barbatula*) in the absence or presence of invasive crayfish species (spinycheek, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) during day and night **b** heatmaps illustrate the localization and dynamics of spinycheek and signal crayfish inside the aquarium during day and night.

Table 4. Mean (±SE) times of different behaviours (minutes) for the benthic fish species *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead) alone (control), or in presence of crayfish (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish) inside and outside (moving or stationary) the shelter during day and night in the tank.

Fish situation		Stone loach			Bullhead		
		Control	Spinycheek	Signal	Control	Spinycheek	Signal
Inside shelter	day	154.7±55	161.0±33	137.4±19	0.0±0.0	218.9±24	69.3±31
	night	41.3±25	67.0±13	56.3±17	0.0±0.0	110.3±23	23.5±13
Outside shelter	moving, day	98.8±32	85.8±19	43.2±18	143.2±30	42.9±13	62.2±21
	moving, night	405.8±18	203.8±20	207.4±45	234.2±108	178.7±25	134.3±34
	stationary, day	105.5±47	113.1±32	179.4±24	216.8±30	98.2±26	228.9±30
	stationary, night	33.0±19	229.2±29	216.2±49	245.8±108	191.0±25	322.2±31

As a result of their inability to occupy the shelter in the presence of crayfish species, the stone loach expressed hiding behaviour (i.e., reduced general activity, lying low). In general, the stone loach was more active at night than the daytime. Such activity was reduced in the presence of both crayfish species, which was indicated by a significantly lower activity index ($F_{2,22} = 7.7$, $P = 0.002$, Fig. 6a, Table 4). During the day, the

Figure 5. Heatmaps (shifting from blue over yellow to red with the increase of time spent in this respective location) is a graphical representation of the shelter occupation and animal distribution in the experimental tank **a** heatmaps showing the movement of bullhead, (*Cottus gobio*) in the absence or presence of invasive crayfish species (spinycheek, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) during day and night **b** heatmaps illustrate the localization and dynamics of spinycheek and signal crayfish inside the aquarium during day and night.

Table 5. Mean (±SE) times of different behaviours (minutes) for the crayfish species *Faxonius limosus* (spinycheek crayfish) and *Pacifastacus leniusculus* (signal crayfish) tracking inside and outside the shelter (moving or stationary) in the presence of *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead) during day and night in the tank.

Fish situation		Stone loach		Bullhead	
		Spinycheek	Signal	Spinycheek	Signal
Inside shelter	day	156.1±21.8	67.2±26.5	62.0±33.4	33.5±21.7
	night	95.2±30.3	26.2±16.1	19.7±18.8	59.4±42.8
Outside shelter	moving, day	109.6±19.7	170.0±33.7	127.2±30.7	174.1±29.6
	moving, night	330.3±35.7	217.0±50.8	389.5±28.3	359.2±34.9
	stationary, day	82.6±14.2	110.0±30.5	139.8±14.9	86.5±30.4
	stationary, night	78.2±15.3	255.7±49.3	102.2±17.5	129.8±36.9

behavioural reaction was less clear, and the activity index was not significantly different ($F_{2,22}$ = 1.9, P = 0.171, Fig. 6b, Table 4). Bullheads spent more time moving inside the aquaria all day (Table 4). This time decreased upon exposure to both crayfish species, especially during the daytime as evidenced by the significantly reduced activity index ($F_{2,21}$ = 4.3., P = 0.02, Fig. 6c). During the night, bullhead's behaviour slightly changed, and the activity index was not significantly different ($F_{2,21}$ = 1. 6, P = 0.216, Fig. 6d).
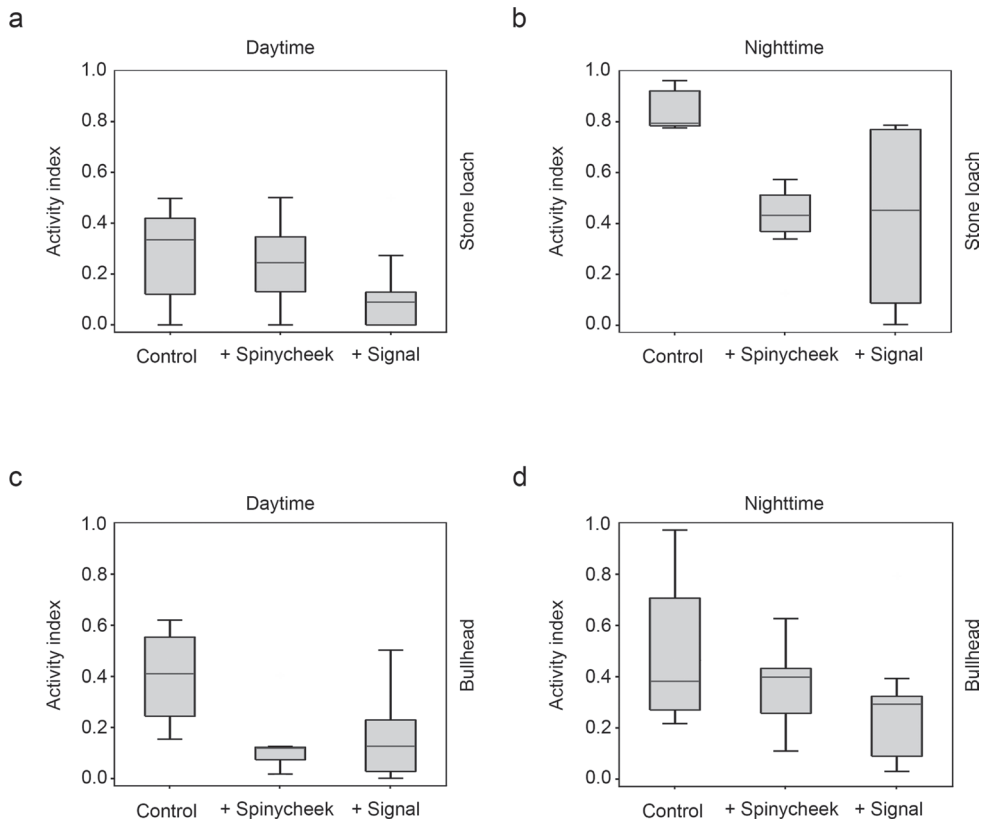
Figure 6. Box plots showing the activity index (*AI*) for the benthic fish species stone loach (*Barbatula barbatula*) (**a, b**) and bullhead (*Cottus gobio*) (**c, d**) in the trials with benthic fish only (Control), in the presence of spinycheek crayfish (*Faxonius limosus*), and in the presence of signal crayfish (*Pacifastacus leniusculus*) during day and night times.

Furthermore, the activity levels of both crayfish species varied, with differences in activity levels observed between the two species as well as the time of the day (Suppl. material 1).

Behavioural interaction

Crayfish species have different levels of agonistic behaviour, with the lowest being an exploratory movement of the antennae and accidentally getting into physical contact with the other animal (non-aggressive interactions) and the highest level being fighting (aggressive interactions). Although there was no statistically significant difference between the two crayfish species in terms of aggressive movement scores towards benthic fish, both species demonstrated aggressive interactions with benthic fish ($F_{1,76}$ = 0.07, p = 0.792). Stone loaches were significantly more exposed to aggressive interactions from both crayfish species than bullheads, as evidenced by the fact that the score of aggressive behaviour was significantly higher ($F_{1,76}$ = 20.5, p < 0.001, Fig. 7a) and the long-lasting interactions ($F_{1,76}$ = 18.8, p < 0.001, Fig. 7b). In addition, crayfish sexes probably affected

the mean score of aggressive movements towards bullheads with the male individuals being more aggressive than females (7.4, 5.3, $F_{1,36}$ = 3.68, p = 0.054, Fig. 7c). For the stone loach, there was no significant difference between crayfish males and females in the mean score of aggressive movements (10.2, 9.85, $F_{1,36}$ = 0.136, p = 0.714, Fig. 7c). The two crayfish species differed markedly with respect to the frequency of the different aggressive acts. Stone loaches were exposed to several aggressive behaviours from both crayfish species, but antennal approaches (score 1) occurred significantly more often than other aggressive acts (Table 6). Half-raised (score 3) or fully raised cheliped (score 4) were the most used aggressive acts by signal crayfish species toward bullheads (Table 6). The frequency of the crayfish aggressive movements increased with the crayfish body mass. In the case of the stone loach, the frequency of aggressive movements correlated positively with carapace length, chela length, and crayfish body mass (r = 0.465, n = 40, p = 0.003; r = 0.560, n = 40, p < 0.001; r = 0.520, n = 40, p < 0.001), respectively (Suppl. material 2). There was no statistically significant relationship between the frequency of aggressive movements and standard length and body mass of stone loach (r = 0.183, n = 40, p = 0.252; r = 0.256, n = 40, p = 0.111), respectively (Suppl. material 2). Accordingly, there were non-significant relationships between the frequency of aggressive movements of crayfish towards bullhead and the carapace length, chela length, crayfish body mass, standard length, and body mass of bullheads (r = 0.119, n = 40, p = 0.464; r = 0.200, n = 40, p = 0.217; r = 0.262, n = 40, p = 0.103; r = 0.027, n = 40, p = 0.869; r = 0.007, n = 40, p = 0.966), respectively (Suppl. material 3). It is worth mentioning that three benthic fish mortalities were recorded due to crayfish aggression within the time frame of the experiment. During the 40 experiments, signal crayfish killed and ate one bullhead and one stone loach, while spinycheek crayfish preyed only on one stone loach.

Table 6. Mann-Whitney test table to estimate the difference between the frequency of different aggressive interactions of invasive crayfish species (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish). Significant results are indicated by bold font.

Aggressive react	Stone loach		Bullhead		Mean Rank			
					Stone loach		Bullhead	
	U	P	U	P	spinycheek	signal	spinycheek	signal
Antenna wave	52.0	**<0.001**	147.5	0.173	27.90	13.10	17.76	22.13
Cheliped touching	150.0	0.176	167.0	0.369	23.00	18.00	22.15	18.85
Cheliped half raise	164.5	0.337	118.0	**0.043**	18.73	22.28	16.21	23.60
Cheliped full raise	189.5	0.775	134.5	**0.055**	21.00	19.98	17.23	23.78
Grappling and pull	187.0	0.541	184.0	0.513	19.85	21.15	19.70	21.30

Discussion

Crayfish and benthic fish often overlap in their niches, showing a high temporal overlap in their needs for shelter (Cooper et al. 2009; Kubec et al. 2019). For crayfish, shelter use can even be of a higher value than access to food (Bergman and Moore 2003) as it lowers the risk of predation and cannibalism (Garvey et al. 1994; Söderbäck 1994; Hill and Lodge 1999; Fero et al. 2007).
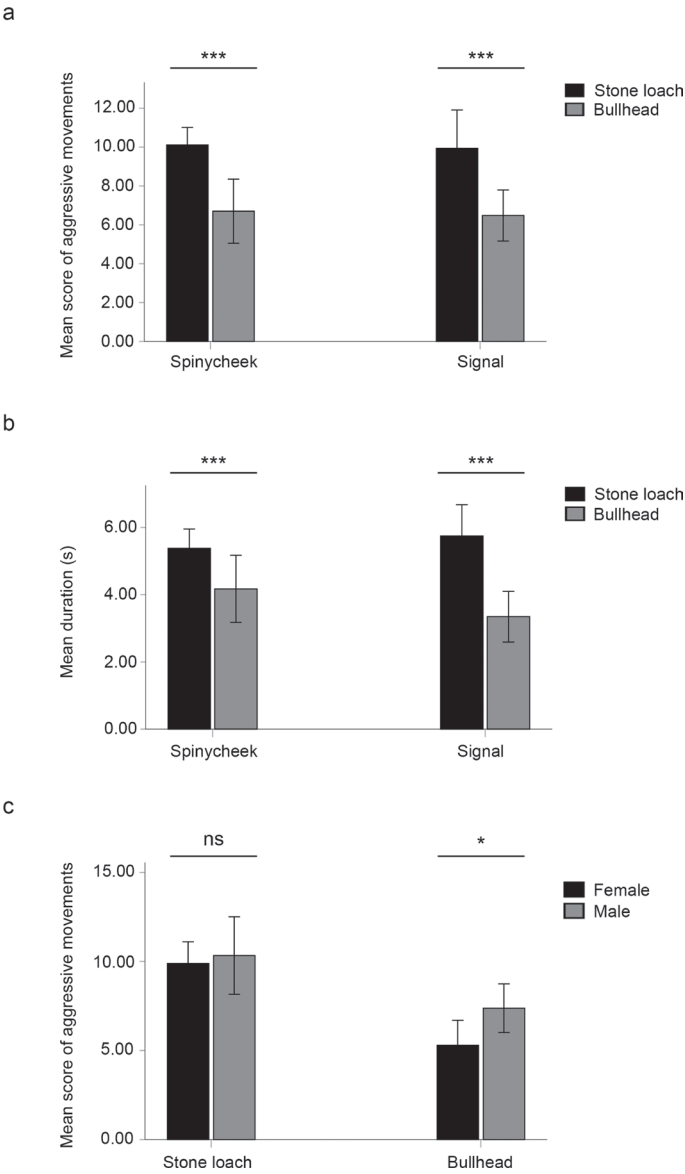
Figure 7. Comparison of aggressive interactions of the two crayfish species (spinycheek crayfish, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) towards the benthic fish species bullhead (*Cottus gobio*) and stone loach (*Barbatula barbatula*). Error bars indicate the standard errors of the mean. (ANOVA; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Our results demonstrate that both stone loach and bullhead, displayed increased hiding behaviour, changing shelter use as well as their activities and movements in the presence of crayfish. Being superior competitors, spinycheek crayfish and signal crayfish succeeded in gaining control of the shelter by displacing stone loaches from the

shelter, thereby decreasing the time of shelter occupation by stone loaches. Bullheads, on the other hand, showed increased shelter use when crayfish were present. This suggests that unlike stone loaches, which avoided the shelter to evade crayfish chasing, bullheads hid inside the shelter. Eviction and displacement of fish species from their own shelter by invasive crayfish under laboratory conditions have been shown not only for small benthic fish like bullheads (Bubb et al. 2009), Paiute sculpin *Cottus beldingi* (Light 2005), cobitid fish *Lefua echigonia* (Matsuzaki et al. 2012), and Johnny darter *Etheostoma nigrum* (Rahel and Stein 1988), but also for bigger and more mobile fish species such as Atlantic salmon *Salmo salar* (Griffiths et al. 2004) and Japanese crucian carp *Carassius auratus* (Matsuzaki et al. 2012). This eviction of fish from the shelters seems to be of ecological relevance as it can markedly increase the risk of direct predation by crayfish or other predators (Rahel and Stein 1988; Matsuzaki et al. 2012).

In addition to the change in shelter use, both benthic fish species showed an increased hiding behaviour in response to the presence of crayfish and because crayfish monopolized the shelter. Fish reduced their general activity and spent long periods stationary at certain places outside the shelter. As a consequence of those behavioural changes, they maintained a greater average distance from the shelter than when crayfish were not present. Perales et al. (2021) showed that virile crayfish (*Faxonius virilis*) shifted away from their habitats as the rusty crayfish (*Faxonius rusticus*) population increased, and paiute sculpin (*Cottus beldingi*) changed their habitat to high-velocity areas in the presence of signal crayfish (Light 2005). These observations of behavioural changes support our interpretation that crayfish are the superior competitors, displacing the benthic fish toward less preferred habitats with fewer competitors.

Our findings demonstrate that both benthic fish species are negatively affected by crayfish invasion, regardless of whether they increased or decreased their shelter use. On the other hand, stone loach is perceived to be more endangered than bullheads. Our data shows that crayfish caused the stone loach to stay away from the shelter to a greater extent than the bullhead. This could imply a more drastic reduction in the stone loach population due to increased susceptibility to predation compared to bullheads. This observation aligns with (MacKenzie and Greenberg 1998), who demonstrated that stone loaches utilized shelters as a means to evade predators.

In addition to shelter and microhabitat use, the aggressive behaviour of invasive crayfish itself can also impact native benthic fish. Highly aggressive behaviour is known to be a key feature of successful invaders (Weis 2010). Furthermore, aggressive dominance is considered an advantage in the competition for shelter use, implying the importance of aggressiveness in replacements among crayfish species (Usio et al. 2001). We observed that both crayfish species displayed several aggressive acts against both species of benthic fish, indicated by the high scores of aggressive interactions. Such antagonistic actions can cause the weaker competitor to change habitat use, and a displacement to suboptimal feeding habitats may impair their population development. This was previously shown for juvenile burbot (*Lota lota*) in Lake Constance exposed to spinycheek crayfish in experimental mesocosms, where burbot was evicted from their preferred shelter (Hirsch and Fischer 2008). Consistently, another

study showed that the native benthic fish Paiute sculpin (*Cottus beldingi*) declined in growth rate due to a reduction of feeding as they spent more time fleeing when signal crayfish was present (Light 2005). Similar effects might be expected for stone loach and bullhead although our experimental arena was not designed to test that kind of behavioural response.

The level of aggression and resource-holding potential of animals can be influenced by many factors such as species, size, age, sex, or energy levels and can therefore serve as the key indicator of crayfish dominance (Marden and Waage 1990; Nijman and Heuts 2000). In this study, we observed that factors such as body mass, carapace length, and chela length enhanced the aggressive behaviour of crayfish against stone loach, while crayfish sex had a more significant impact in case of bullheads. These findings are consistent with observations made for other fish species (Figler et al. 1995; Huber and Schroeder 2001; Daws et al. 2002; Klocker and Strayer 2004; Fero et al. 2007; Moore 2007; Patullo et al. 2009). This was also consistent with the reported influence of these factors on the outcome and aggression level in animal fights in general (Marden and Waage 1990; Edsman and Jonsson 1996; Nijman and Heuts 2000).

Previously reported gut content analyses showed that crayfish can feed on bullheads (Guan and Wiles 1998). In line, another study showed that two bullhead mortalities were observed upon exposure to signal crayfish under lab conditions (Bubb et al. 2009). We noted three benthic fish mortalities due to the aggressive behaviour of crayfish within the time frame of our study. Our data suggest that fish mortalities due to the direct predation by crayfish or because of the constitutive stress caused by the presence of crayfish can serve as a relevant factor for fish density.

We conclude that the remarkable dominance of the invasive crayfish species over benthic fish, which we observed under laboratory conditions, such as displacing them from the shelter and changing their behaviour, can render them susceptible to predation. Furthermore, both invasive crayfish species exhibit aggression towards benthic fish.

Acknowledgements

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Supplementary material 1

Activity index (*AI*) box plots
Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann
Data type: image (.png file)
Explanation note: Box plots showing the activity index (*AI*) for the crayfish species signal (*Pacifastacus leniusculus*) (a, b) and spinycheek (*Faxonius limosus*) (c, d) in the trials with benthic fish bullhead (*Cottus gobio*) and stone loach (*Barbatula barbatula*) during the day and night times.

Link: https://doi.org/10.3897/neobiota.83.102975.suppl1

Supplementary material 2

Scatterplot of the frequency of crayfish aggressive movements vs. carapace length, chela length, body mass, stone loach standard length, and stone loach body mass

Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann

Data type: image (.png file)

Explanation note: Data for both crayfish species were pooled. a-c) Scatterplots show aggression towards stone loaches which was positively correlated with carapace length (r=0.465, p=0.003), chela length (r=0.560, p<0.001), and crayfish body mass (r=0.520, p=0.001).

Link: https://doi.org/10.3897/neobiota.83.102975.suppl2

Supplementary material 3

Correlation scatterplot of the crayfish frequency of aggressive movements, carapace length, chela length, body mass, bullhead standard length, and bullhead body mass

Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann

Data type: image (.png file)

Explanation note: Data for both crayfish species were pooled. a-e) Scatterplots display aggression towards bullhead.

Link: https://doi.org/10.3897/neobiota.83.102975.suppl3

RESEARCH ARTICLE

Searching for the competitive ability of the alien seagrass *Halophila stipulacea* with the autochthonous species *Cymodocea nodosa*

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Abstract

The tropical seagrass *Halophila stipulacea* (Forsskål) Ascherson, 1867 entered in the Mediterranean Sea through the Suez Canal more than 100 years ago. In coastal-marine ecosystems the spatial niche of *H. stipulacea* is often overlapped with that of native Mediterranean Sea seagrasses and therefore it might out-compete them. Aiming to better understand its invasiveness potential, we monitored a Southern Mediterranean shallow coastal-marine water habitat from August 2010 to August 2011, where *H. stipulacea* co-occurred with the native seagrass *Cymodocea nodosa* (Ucria) Ascherson, 1870. Besides, the year-round dynamics of *H. stipulacea* was also monitored in four periods. To test the hypothesis that the presence/absence of *H. stipulacea* may have an effect on *C. nodosa* density, we analyzed the shoot density of *C. nodosa* in 8 sites, 4 sites where *H. stipulacea* was present (impacted sites) and 4 where *H. stipulacea* was absent (control sites). The results showed significant differences in *C. nodosa* shoot density according to the presence/absence of *H. stipulacea*, with the lowest values observed in sites where it co-occurred with *H. stipulacea*. We hypothesize that the dense rhizome-sediment net created by *H. stipulacea* can interfere with *C. nodosa* density, pushing down its rhizomes in the anoxic layer. The leaf features of *H. stipulacea* were generally comparable to those of other Mediterranean populations. In January 2011 a significant decline of *H. stipulacea* was observed, maybe related to changes in the environmental conditions that have

become unfavorable (e.g. hydrodynamics, turbidity) and, unexpectedly, the seagrass disappeared in April 2011. In January, we also observed the occurrence of the green alien alga *Caulerpa cylindracea* Sonder, 1945 which rapidly invaded the bare substrate left by *H. stipulacea*.

Keywords

Cymodocea nodosa, *Halophila stipulacea*, invasive alien species (IAS), Mediterranean Sea, non-indigenous species (NIS), seagrasses, shallow coastal-marine habitat

Introduction

Alien or non-indigenous species (NIS, i.e. organisms introduced from beyond their natural, past or present, geographical region and outside of their natural dispersal potential) are widely recognized as a major threat to native biodiversity, ecosystem functioning and services (Wallentinus and Nyberg 2007; Katsanevakis et al. 2014; Vergés et al. 2016). Besides that, they may in time become invasive (Invasive Alien Species "IAS"), determining significant environmental, socio-economic and human health impacts, such as biodiversity loss and ecosystem services degradation (Giakoumi 2014; Gallardo et al. 2016; Vergés et al. 2016). The Mediterranean Sea is a hot-spot of biodiversity (e.g. Coll et al. 2010), but the dramatically-accelerating rate of alien species introduction made it also a true hot-spot for biological invasions (Zenetos et al. 2017; Galil et al. 2018; Bariche et al. 2020; Zenetos and Galanidi 2020).

Islands, also considered hotspots of biodiversity, are vulnerable to anthropogenic pressures as well as for hosting NIS (Tershy et al. 2015; Domina et al. 2018). In particular, Sicily Island and circum-Sicilian Islands (Central Mediterranean Sea, Italy) are notably prone to biological invasions, due to their strategic position at the crossroads between the South Western and Eastern Mediterranean Sea, by virtue of the intense maritime traffic skirting the region and lessepsian migrations (Occhipinti-Ambrogi et al. 2011a, 2011b; Katsanevakis et al. 2014; Mannino et al. 2014, 2017, 2018, 2019).

Among the NIS entered in the Mediterranean Sea, there is the putative lessepsian migrant (tropical species that migrate into the Mediterranean Sea through the Suez Canal) *Halophila stipulacea* (Forsskål) Ascherson, 1867 (Hydrocharitaceae), a small seagrass native of the Red Sea, Persian Gulf, and Indian Ocean (Lipkin 1975; El Shaffai 2011). It is the only alien seagrass entered in the Mediterranean Sea given that the *Halophila* population found in Salamina Island, Greece (initially indicated as *Halophila decipiens* Ostenfeld, 1902), most likely corresponds to a morphological variant of *H. stipulacea* (Gerakaris et al. 2020; García-Escudero et al. 2022). *Halophila stipulacea*, reported for the first time in the Mediterranean Sea off the Rhodes Island coast (Fritsch 1895), spread along the Eastern Mediterranean Sea and progressively expanded westwards and northwards (see Gambi et al. 2009, 2018; Tsiamis et al. 2010; Katsanevakis 2011; Sghaier et al. 2011, 2019; Varela-Álvarez et al. 2011; Winters et al. 2020; Di Genio et al. 2021; Hoffman 2021; Pica et al. 2021; Thibaut et al. 2022), mostly invading shallow water locations close to ports and marinas, suggesting that maritime traffic was the main vector of spreading. Currently, Cannes (France) represents the most

northern location in the Western Mediterranean Sea, suggesting a rapid expansion of this species likely due to climate change and tropicalization. The species also entered in the tropical Atlantic Ocean (Caribbean Sea) where it has been rapidly expanding (Winters et al. 2020), and it is assumed that this genotype came from the Mediterranean Sea (Ruiz and Ballantine 2004; Boudouresque et al. 2016).

Halophila stipulacea is generally considered a relative fast-growing seagrass, and its success is attributed to its high morphological, physiological and biochemical plasticity and ability to spread and adapt to a wide range of environmental conditions (Gambi et al. 2009; Willette and Ambrose 2009; Mejia et al. 2016; Oscar et al. 2018; Beca-Carretero et al. 2020; Wesselmann et al. 2020; Winters et al. 2020; Thibaut et al. 2022). According to Oscar et al. (2018), the invasive nature of *H. stipulacea* in the Mediterranean and Caribbean Sea could be explained, in particular, by its remarkable tolerance to a wide range of temperatures (14–36 °C) and salinities (from 25 PSU to 60 PSU). Furthermore, Wesselmann et al. (2020) provide evidence for an upper thermal tolerance of *H. stipulacea* higher than those of the native seagrasses, particularly *Posidonia oceanica* (L.) Delile, 1813. Thus, the increase of seawater temperature in the Mediterranean Sea would improve the *H. stipulacea* performance (growth, recruitment and metabolic rates) but also its expansion into the Western Mediterranean basin (Wesselmann et al. 2020; Di Genio et al. 2021).

Moreover, Rotini et al. (2017) showed that the associated bacterial consortium may help *H. stipulacea* to better cope with environmental changes, thus having an important role in its ecological resilience and invasiveness. Additionally, its ability to acquire ammonium efficiently either from the water or from the sediment, may confer it an advantage with respect to other Mediterranean seagrasses (Alexandre et al. 2014).

As a result of its high tolerance, it has been estimated that in the near future *H. stipulacea* will be present throughout the whole Mediterranean Sea (Georgiou et al. 2016; Beca-Carretero et al. 2020; Wesselmann et al. 2020). The indigenous warm-temperate seagrass *Cymodocea nodosa* (Ucria) Ascherson, 1870, is also able to tolerate high temperatures (34–35 °C, optimum temperature at 30 °C) and a wide range of salinities (between 10 and 50 PSU) (Tsioli et al. 2019). Under climate change scenarios, it is therefore expected *C. nodosa* to cope better with environmental changes, providing it with an advantage compared with *P. oceanica*.

In the Mediterranean Sea, *H. stipulacea* frequently co-occurs with native seagrasses such as *C. nodosa*, *P. oceanica*, *Zostera noltei* Hornemann, 1832 and native or introduced macroalgae such as *Caulerpa prolifera* (Forsskål) Lamouroux, 1809, *Caulerpa cylindracea* Sonder, 1845, and *Caulerpa taxifolia* var. *distichophylla* (Sonder) Verlaque, Huisman & Procaccini, 2013 (Kashta and Pizzuto 1995; Di Martino et al. 2006; Sghaier et al. 2011; Gaglioti and Gambi 2018). Although *H. stipulacea* is listed among the worst invasive species (Boudouresque and Verlaque 2002; Streftaris and Zenetos 2006), there is little evidence of its impact on native macrophytes in the Mediterranean Sea (Tsiamis et al. 2010; Sghaier et al. 2014; Hoffman 2021; Tsirintanis et al. 2022; Conte et al. 2023). Tsirintanis et al. (2022), for instance, reported for *H. stipulacea* a medium strength of evidence of combined negative and positive impacts on biodiversity. While Hoffman (2021) stated that the invasion of *H. stipulacea* into the sub-tidal of the Levant Mediter-

ranean coast of Israel may represent a serious threat to the scarce population of *C. nodosa* down into the deep sub-tidal, as a consequence of the increased seawater temperatures. Moreover, Kolátková et al. (2021) hypothesized that a fungal parasite, recently discovered on Mediterranean *H. stipulacea*, may also affect native seagrasses such as *C. nodosa*. Recently, Conte et al. (2023) highlighted the potential capability of *H. stipulacea* to outcompete the native *C. nodosa* in the Aegean Sea, related to its morphophysiological plasticity and the ability to harbor and interact with diversified bacterial communities.

Instead, in the Caribbean Sea where it has been recently introduced, *H. stipulacea* is rapidly displacing native seagrasses (e.g. *Syringodium filiforme* Kützing, 1860) (Willette and Ambrose 2009, 2012; Willette et al. 2014; van Tussenbroek et al. 2016).

Since the ongoing tropicalization pattern of the Mediterranean Sea could facilitate *H. stipulacea* to compete with native seagrasses such as *C. nodosa* (Chefaoui et al. 2018; Savva et al. 2018; Wesselmann et al. 2020), it is mandatory to better understand the role of this alien species within native communities. Hence, we tested the hypothesis that the presence/absence of *H. stipulacea* may have an effect on native seagrasses. To do that, we surveyed from August 2010 to August 2011 a Southern Mediterranean shallow water habitat where *H. stipulacea* co-occurred with *C. nodosa*. In particular, the shoot density of *C. nodosa* in sites where *C. nodosa* co-occurred with *H. stipulacea* (impacted sites) and sites where *C. nodosa* formed monospecific populations (control sites) were analyzed. As information regarding the year-round dynamics of *H. stipulacea* in the Mediterranean Sea is scarce (Nguyen et al. 2020; Di Genio et al. 2021), we also monitored over four periods the *H. stipulacea* biometric features.

Materials and methods

Study area

The study was carried out in semi-artificial shallow water basins located in proximity to the harbour of Termini Imerese (Fig. 1; 37°59'00"N, 13°42'00"E; North-Western Sicily, Italy, Southern Mediterranean Sea). To date, this is the only location in North-Western Sicily where *H. stipulacea* records have been reported (Mannino et al. 2009). The basins (hereafter referred to as sites) range in size from 5,500 m² to 10,000 m² and are connected to the sea via a central opening. The environmental characteristics at the sites are comparable, with sandy bottoms and depths ranging from 0.8 to 2.5 meters. Salinity remained consistent (38) throughout the seasons, while temperatures ranged from 25 °C in summer to 13 °C in winter (Table 1). Preliminary surveys revealed that *H. stipulacea* is not present in all sites but becomes dominant in those nearest to the harbor, whereas *C. nodosa* increases its presence into sites further away from the harbor. However, it is not clear if the presence of *H. stipulacea* can affect the density of *C. nodosa* or if the two species can co-occur. Due to the co-occurrence of these two species in this location, as well as the similar environmental characteristics, these sites are natural mesocosms that provide a unique opportunity to investigate the interaction of *H. stipulacea* and *C. nodosa*.
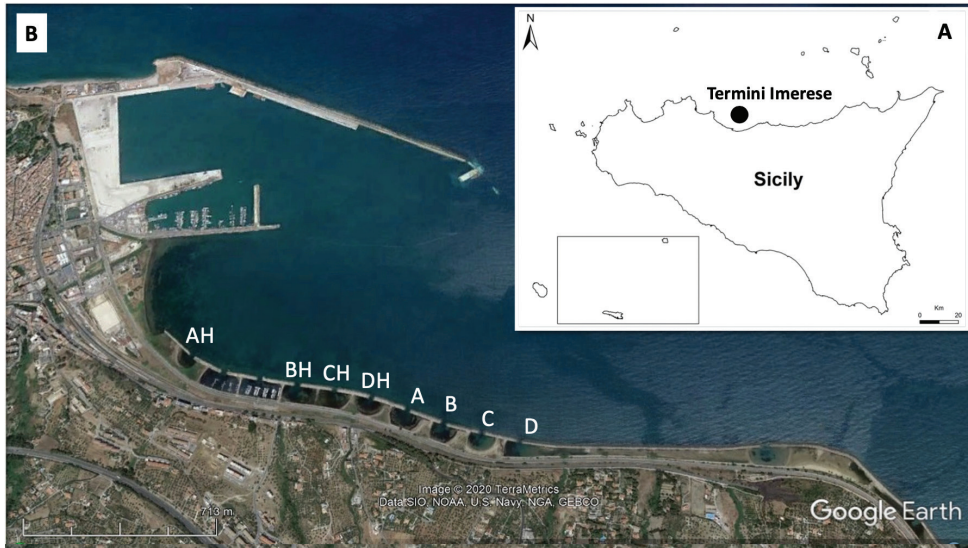
Figure 1. The study area (**A**) and details showing the investigated sampling sites (**B**). Impacted sampling sites (*Halophila stipulacea* present) are listed as: AH, BH, CH, DH. Control sampling sites (*Halophila stipulacea* absent) are listed as: A, B, C, D.

Table 1. Mean values (± SE) of measured environmental factors at the sampling sites in four periods (T1 = August, T2 = October, T3 = January, T4 = April).

Abiotic features	T1	T2	T3	T4
Temperature (°C)	24.89 ± 0.23	21.97 ± 0.16	13.97 ± 0.16	17 ± 0.12
Salinity (PSU)	38.21 ± 0.07	38.10 ± 0.06	38 ± 0.08	38 ± 0.07

Experimental design

The experiment was carried out at 8 sites. In particular, four sites (A, B, C, D; control sites) characterized by the presence of monospecific *C. nodosa* populations and four sites (AH, BH, CH, DH; impacted sites) where *C. nodosa* co-occurred with *H. stipulacea*. Sites were surveyed from August 2010 to August 2011 and samplings were carried out in four periods (T1 = August, T2 = October, T3 = January, T4 = April).

The shoot density (number of shoots/m²) of *C. nodosa* and *H. stipulacea* was estimated by counting the number of shoots present in 3 randomly located quadrats (20 × 20 cm). The sampled shoots were brought to the lab, then were washed with seawater, sieved to remove sediment and big debris, and ultimately stored in labelled bags at 4 °C. For each site, the biometric features of *H. stipulacea* were then estimated by measuring the length and width of 30 randomly selected leaves (+/- 1 mm) in triplicate. To collect plant samples a formal permission was not required. Representative plant samples were deposited in the algological laboratory of the Department STeBiCeF - University of Palermo, Italy.

Statistical analysis

Differences in the density of *C. nodosa* among periods (fixed and orthogonal with 4 levels; T1, T2, T3 and T4), conditions (fixed and orthogonal with 2 levels; control vs impacted), sites (random and nested within conditions with 4 levels; 1, 2, 3 and 4) and their interaction were assessed using analysis of variance (ANOVA). Cochran's test was used to check for the homogeneity of variances (Underwood 1996).

To investigate which factor explained the variation in *C. nodosa* density, general mixed models (GLMs) were built using the "lme" function of the R package "nlme" (Pinheiro et al. 2016). These models included the density of *H. stipulacea*, temperature, and salinity as independent fixed continuous variables, the time as an independent random factor, and the density of *C. nodosa* as continuous dependent variable. When a variable did not follow normal distribution, it was log transformed to reach normal distribution before statistical analyses. We chose the best model for each dependent variable based on the Akaike information criterion, and we used the R package "MuMIn" (Barton 2012) to estimate variance explained by the mixed models. The number of degrees of freedom was determined using the "Kenward-Roger" method. All interactions up to the third level have been tested.

A descriptive analysis of *H. stipulacea* was carried out by calculating average (± SE) length and width of leaves, as well as its shoot density. ANOVA models were performed to investigate possible relationships between the measured variables (temperature, salinity, time, and *C. nodosa* densities) and width and length of *H. stipulacea* leaves.

Results

The *C. nodosa* shoot density was affected by the presence of *H. stipulacea*, with densities significantly lower in impacted sites compared to control ones (Fig. 2, Table 2). This effect was consistently observed through all investigated periods, although the density of *C. nodosa* tends to decrease from August 2010 to April 2011, with differences more marked in control sites than impacted ones (Fig. 2, Table 2).

Table 2. ANOVA results for testing the effects of sampling times and treatments on the density of the autochthonous species *Cymodocea nodosa*. Data were tested with the Cochran test (C=0.19; P>0.05) and then log transformed. Level of significance "P": *** <0.001; ** <0.05, * <0.1.

	DF	MS	F	P
Time	3	0.63	940	***
Treatment	1	13	19000	***
Time*Treatment	3	0.67	990	***
Treatment*Site	6	0.0014	2.1	*
Time*Treatment*Site	18	0.0014	2	**
Residuals	64	0.00068		
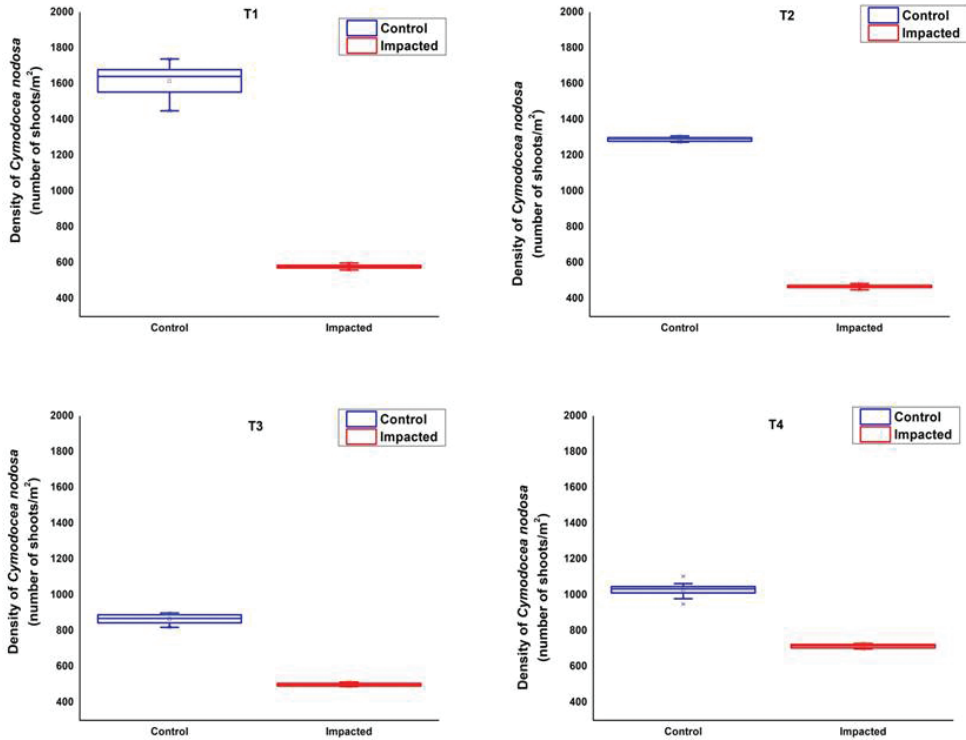
Figure 2. Variation of *C. nodosa* density in control sampling sites and in impacted sampling sites at each sampling time (T1 = August, T2 = October, T3 = January, T4 = April). As a preliminary analysis at each time, the sampling sites of each treatment were pairwise compared through a one-way ANOVA. No significant differences were found among sampling sites within the two "control" and "impacted" groups at each sampling time. The box plots were built merging the observations of each sampling site for each sampling time and treatment.

Plant interaction assessment

Shoot density of *H. stipulacea*, temperature, their interaction, as well as the interaction between *H. stipulacea* density, temperature and salinity were significant (Table 3).

At level of single predictor only the variables *H.* Density and Temperature result significant; the two variables result with negative parameters according to an inverse relationship. Consistently with the results of the ANOVA for the decomposition of the variance, the parameters of *H. stipulacea* density, temperature, their interaction and the interaction between *H. stipulacea* density, temperature and salinity were significant (Table 4).

The analysis of the first level of interaction showed that *C. nodosa* shoot density was negatively related to the increase of *H. stipulacea* density (Fig. 3A, see also Fig. 4A–C), while showing a positive relationship with temperature and salinity (Fig. 3B, C).

Table 3. Type III Analysis of Variance Table. The table reports the sum of squares, mean square, degrees of freedom of numerator and denominator, F value and Variance ratio (Pr) for each fixed independent variable and for each considered interaction (in bold significant effects). "*H.* Density" indicates the shoot density of *H. stipulacea*.

	SS	MS	NumDF	DenDF	F value	Pr(>F)
H. **Density**	27914.595	7424.866	1.000	85.901	0.811	**0.870**
Temperature	120933.622	10872.959	1.000	87.740	1.187	**1.279**
Salinity	9856.924	9856.924	1.000	1.925	1.077	0.412
H. **Density*Temperature**	19882.804	9149.827	1.000	85.890	0.099	**0.320**
H. **Density*Salinity**	7957.338	7957.338	1.000	85.895	0.369	0.354
Temperature*Salinity	385561.246	385561.246	1.000	1.954	42.109	0.024
H. **Density*Temperature*Salinity**	31951.772	14124.101	1.000	85.798	0.543	**0.718**

Table 4. ANOVA table of fixed factors. The table shows the estimated values of the fixed factors, the standard error, the degrees of freedom and the significance values for each fixed factor and for each considered interaction (in bold significant effects). "*H.* Density" indicates the shoot density of *H. stipulacea*.

	Estimate	Std. Error	DF	t value	Pr(>|t|)
(Intercept)	52631.390	24667.133	87.982	2.134	0.036
H. **Density**	-7.227	11.250	85.813	-0.642	**0.722**
Temperature	-2195.345	1397.647	87.558	-0.571	**0.620**
Salinity	-1389.506	646.783	87.994	-2.148	0.034
H. **Density*Temperature**	0.257	0.482	85.982	0.534	**0.595**
H. **Density*Salinity**	0.183	0.296	85.811	0.618	0.538
Temperature*Salinity	59.563	36.622	87.629	1.626	0.107
H. **Density*Temperature*Salinity**	-0.007	0.013	85.977	-0.517	**0.606**
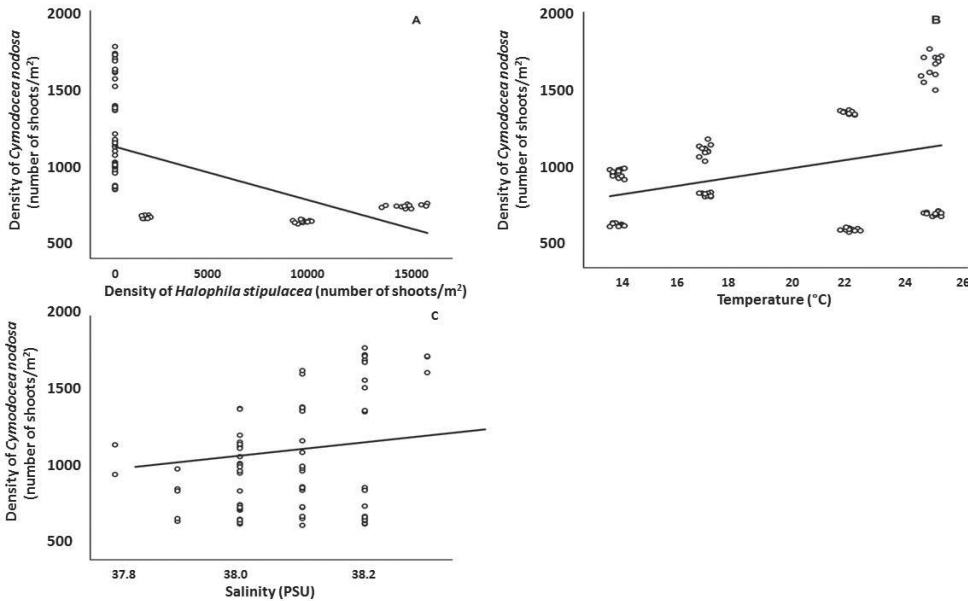
Figure 3. Relationship between *C. nodosa* density and *H. stipulacea* density (**A**), temperature (**B**) and salinity (**C**).
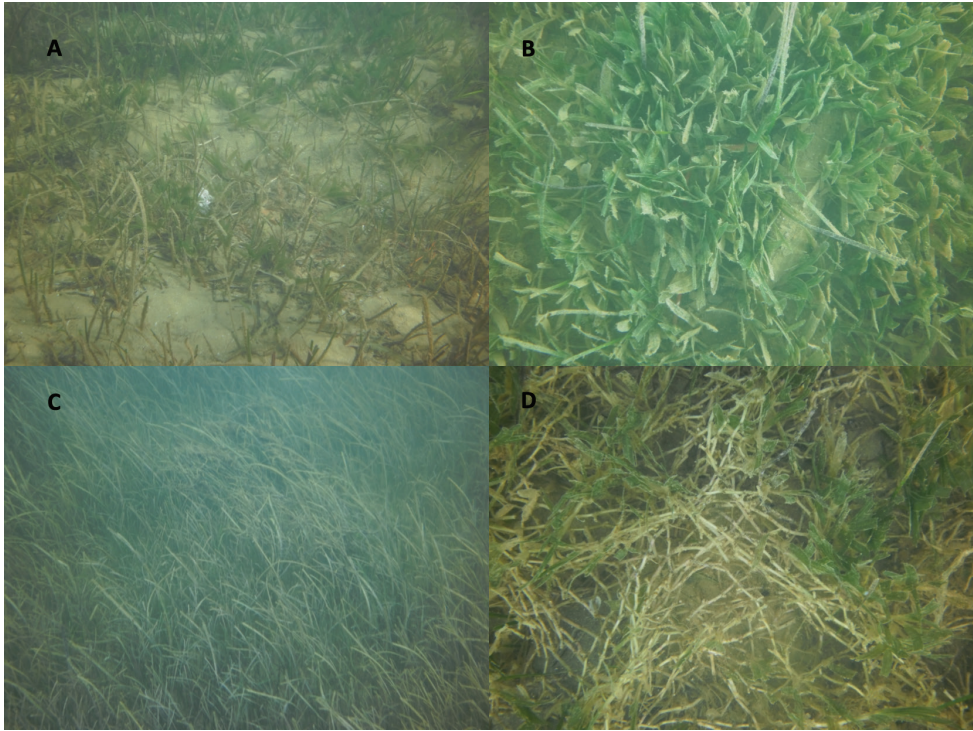
Figure 4. Examples of habitat structure at the investigated sites. *Cymodocea nodosa* in presence of *Halophila stipulacea* (**A**), *Halophila stipulacea* dominating *Cymodocea nodosa* (**B**), *Cymodocea nodosa* in absence of *Halophila stipulacea* (**C**), multi-layered mat formed by rhizomes of *Halophila stipulacea* (**D**).

We observed a dense multi-layered mat formed by the lateral rhizomes of *H. stipulacea*, growing between *C. nodosa* shoots and entrapping sediment (Fig. 4D). Therefore, in control sites rhizomes of *C. nodosa* grew above the sediment or were slightly covered by it (Fig. 5A) while in impacted sites they were pushed down in the anoxic layer by the multi-layered mat (Fig. 5B).

The second level interactions, on the other hand, produced negative relationships for the interaction between *H. stipulacea* density and temperature (Fig. 6A), and between *H. stipulacea* density and salinity (Fig. 6B); while the interaction between temperature and salinity showed a positive relationship with the density of *C. nodosa* (Fig. 6C). The third level interaction shows negative relationships for all the considered conditions (Fig. 6D).

The results of the mixed model clearly showed 3 different effects of the considered independent variables on *C. nodosa* density values. Temperature had a positive effect, *H. stipulacea* density had a negative effect and salinity (in the recorded range of values) showed a conservative effect. Their interactions clearly showed the strength of the interaction with the presence of *H. stipulacea*, a relationship appearing limited by the temperature that functions as a control variable on the negative effect led by the density of *H. stipulacea* (Fig. 6A, D).
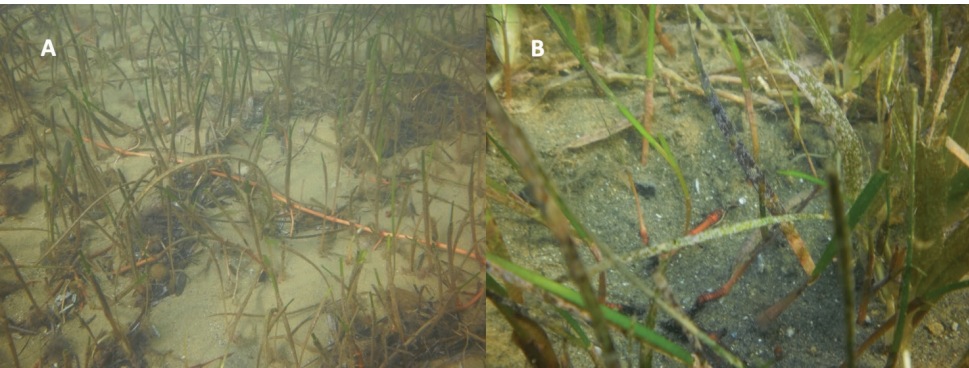
Figure 5. *Cymodocea nodosa*: rhizomes above the sediment (**A**), rhizomes pushed down in the anoxic layer (**B**).
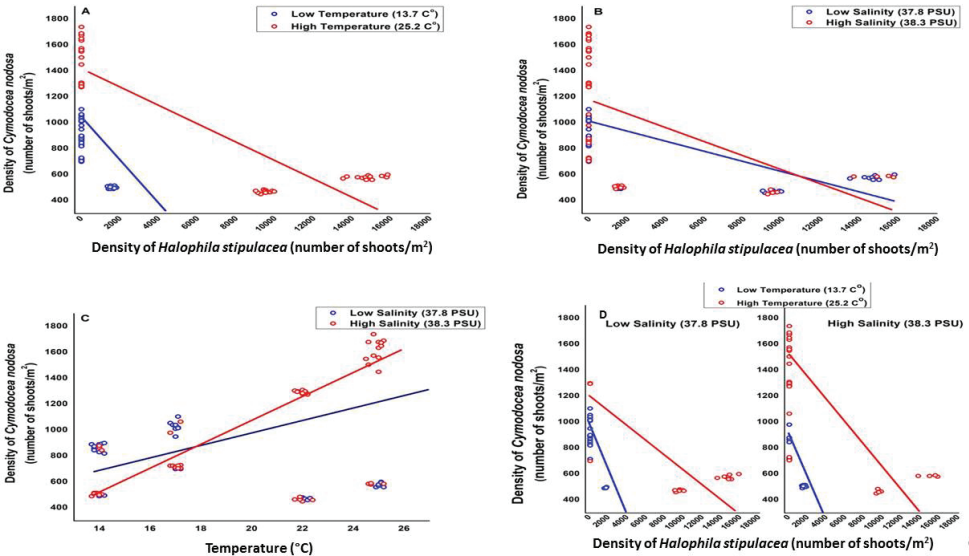
Figure 6. Plot of the second (**A–C**) and third (**D**) level interactions, the figure shows the plot of the relationship resulting from the interaction between *H. stipulacea* density and temperature (**A**), *H. stipulacea* density and salinity (**B**), temperature and salinity (**C**) and for the interaction between all the fixed factors (**D**).

Biometric features of *H. stipulacea*

Biometric features of *H. stipulacea* are reported in Fig. 7A, B. Elliptic leaf blades, typically characterized by a distinct mid vein with many branched cross veins, were long on average 59.0 mm ± 1.8 (specifically, 59.1 mm ± 1.6 in T1, 58.8 mm ± 1.8 in T2 and 59.2 mm ± 1.8 in T3) and wide on average 6.8 mm ± 0.175 (6.921 mm ± 0.15 in T1, 6.8 mm ± 0.16 in T2 and 6.7 mm ± 0.20 in T3; Table 5). Leaf length and width didn't show significant differences among sites and sampling periods (P>0.05; Fig. 7A, B). Shoot density of *H. stipulacea* showed a mean value of 8,613.33 ± 384.31 number of shoots/m² and a consistent reduction from T1 to T3
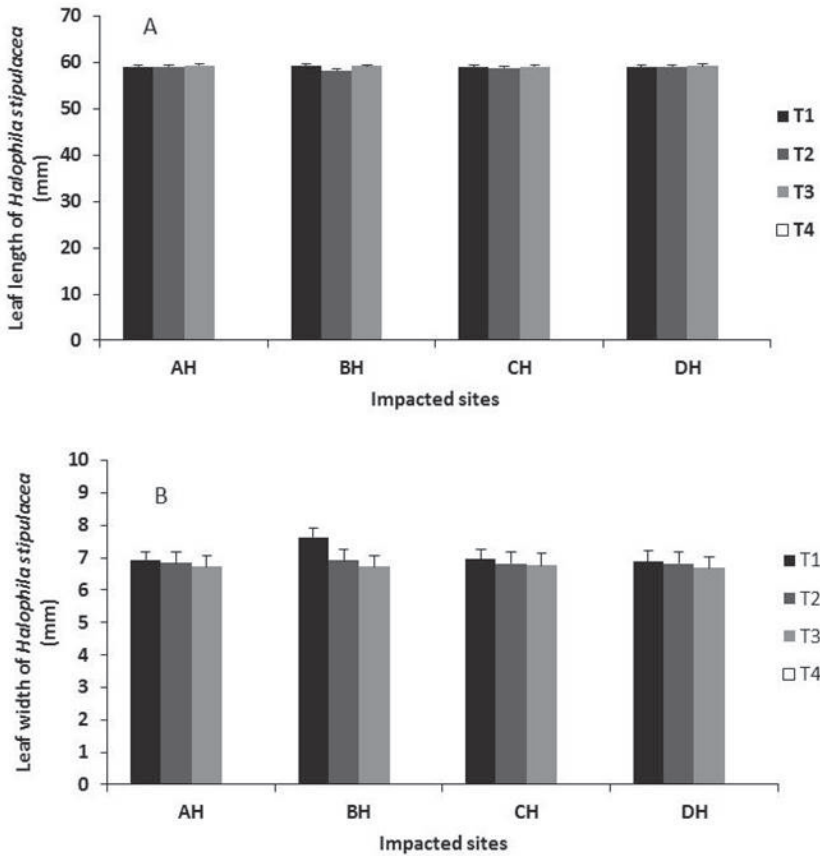
Figure 7. Leaf length (**A**) and leaf width (**B**) of *H. stipulacea* in impacted sampling sites. Bars show mean ± SE (n = 30). In T4 the species disappeared.

was observed (14,754.17 ± 722.51 in T1, 9,495.83 ± 287.98 in T2, and 1,590.0 ± 142.45 in T3). In T4 *H. stipulacea* totally disappeared except for the occurrence of a few dead shoots. Flowers have never been observed. Notably, we observed the occurrence in T3 of *C. cylindracea* (cover of 2.5%), that rapidly invaded the bare substrate left by *H. stipulacea*, reaching a cover of 40% in T4. Surveys carried out in August 2011 confirmed the presence of *C. cylindracea*, which reached a cover of about 70%.

As the best three-way ANOVA model for the "leaf width" variable, the model composed by the variables time, temperature and *C. nodosa* density was selected, based on the values of R^2 (0.993) the model explains 99% of the observed variability. The best three-way ANOVA model built for the "leaf length" variable is instead the model composed of the categorical variable "Time", based on the values of R^2 (0.931) the model explains 93% of the observed variability. In both cases based on the values of the Fisher statistic (F), the information brought by the explanatory variables is significantly better than a basic mean would bring (Table 6).

For each selected model the interactions up to the third level were evaluated; in both models the only highly significant variable is time, the variables temperature, salinity and *C. nodosa* density are weakly significant (Table 7). The interactions temperature*time, salinity*time, *C. nodosa* density*time and *H. stipulacea* density*time are weakly significant (Table 7); while all other variables and other interactions are not significant.

Table 5. Biometric data (mean ± SE) of *H. stipulacea* in the Mediterranean Sea.

Localities	References	Depth (m)	Mean density (No. of shoots/m²)	Mean leaf length (mm)	Mean leaf width (mm)
Termini Imerese harbor (Italy)	Present study	0.8 – 2.5	8,613.3 ± 384.31	59.07 ± 1.80	6.83 ± 0.17
Palinuro harbor (Italy)	Gambi et al. (2009)	2 – 5	10,500 ± 2,700	33.3 – 55.7	4.4 – 6.8
	Di Genio et al. (2021)	1.8 – 4	from 6,100 ± 953.9 to 9,290 ± 2,482	25.0–50.0	4.5 – 7.0
Peninsula of Maddalena (Italy)	Di Martino et al. (2006)	21	1,967	42 – 73	m.d.
Vulcano Island (Italy)	Procaccini et al. (1999)	5 – 25	12,795 – 15,170	40.3 – 67.5	5.1 – 7.8
Oliveri-Tindari (Italy)	Procaccini et al. (1999)	2	25,345 ± 4,324	63.8 – 84.3	8.3 – 10.1
Naxos-Taormina (Italy)	Cancemi et al. (1994)	2	19,728	m.d.	m.d.
Marina Cap Monastir (Tunisia)	Sghaier et al. (2011)	1 – 2	9,900 ± 3,509	58.2 ± 4.3	7.1 ± 0.7
Tobrouk Bay (Libya)	Sghaier et al. (2011)	1 – 1.5	476 ± 83	47	55
Cannes (France)	Thibaut et al. (2022)	11 – 17	202	up to 57	m.d.

m.d. = missing data**.**

Table 6. ANOVA table of the two computed three-way ANOVA models. WModel and LModel indicate the model constructed for the variable "*H. stipulacea* leaf width" and "*H. stipulacea* leaf length" respectively.

Source	DF	SS	MS	F	Pr > F	P
WModel	13.000	428.876	32.990	354.997	**<0.0001**	***
Error	34.000	3.160	0.093			
Corrected Total	47.000	432.036				
LModel	7.000	31405.311	4486.473	88002.034	**<0.0001**	***
Error	40.000	2.039	0.051			
Corrected Total	47.000	31407.351				

Level of significance codes (P): 0 < *** < 0.001.

Discussion

Although *H. stipulacea* is listed among one of the worst invasive species (Streftaris and Zenetos 2006), there is no clear evidence of competition with Mediterranean seagrasses, possibly due to its small shoot size compared to that of larger native species (Apostolaki et al. 2019). The co-occurrence of *H. stipulacea* and *C. nodosa* has been already reported for the Mediterranean Sea but negative interactions between the two seagrasses were not clearly observed except for the Tunisian and Crete (Greece) coasts (Kashta and Pizzuto 1995; Di Martino et al. 2006; Sghaier et al. 2014; Conte et al. 2023).

Table 7. Type III Analysis of Variance. The table reports the values of degrees of freedom, sum of squares, mean squares, F statistic and P-value for the factors and the interactions which resulted at least scarcely significant (0.1 < * < 1). Factors and interactions not present were found to be insignificant. Significant interactions are reported.

Source	DF	SS	MS	F	Pr > F	P	DF	SS	MS	F	Pr > F	P
	leaf length						leaf width					
Temperature	1.000	0.009	0.009	0.145	0.706	*	1.000	0.030	0.030	0.324	0.573	*
Salinity	1.000	0.037	0.037	0.630	0.433	*	1.000	0.069	0.069	0.748	0.393	*
C. nodosa Shoot density	1.000	0.019	0.019	0.316	0.578	*	1.000	0.006	0.006	0.066	0.798	*
Time	3.000	0.905	0.302	5.122	**0.005**	**	3.000	0.334	0.111	0.041	**0.324**	**
Temperature*Time	3.000	0.023	0.008	0.132	0.940	*	3.000	0.476	0.159	1.713	0.183	*
H. stipulacea density*Time	3.000	0.962	0.321	5.444	**0.004**	**	3.000	0.258	0.086	0.931	0.437	*
C. nodosa density *Time	1.000	0.017	0.017	0.282	0.599	*	1.000	-0.338	-0.338	-3.655	1.000	*

Level of significance codes (P): 0.001 < ** < 0.01, 0.1 < * < 1.

Sghaier et al. (2014) reported a relevant competition between *C. nodosa* and *H. stipulacea* in Tunisian coast, with *H. stipulacea* displacing till 50% of *C. nodosa*. In Crete, Conte et al. (2023) highlighted severe signs of stress (altered associated bacterial communities, biometric and biochemical descriptors) in *C. nodosa* due to the proximity of *H. stipulacea*. In the Caribbean Sea, instead, a significant competition for space was reported between *H. stipulacea* and the native seagrass *S. filiforme*, with *H. stipulacea* rapidly expanding and displacing the local seagrass but also altering the seagrass community (Willette and Ambrose 2012).

The observed values of shoot density and the total absence of flowers and/or fruits in *C. nodosa* in impacted sites might be linked to a negative effect of *H. stipulacea* on *C. nodosa* growth. The system outlined by the physical conditions (temperature and salinity) and the presence of *H. stipulacea*, analyzed through a mixed model approach, showed different effects on the density of *C. nodosa*. The temperature is the main favoring factor with respect to the density of *C. nodosa* while the density of *H. stipulacea* has a strongly limiting effect on the density of *C. nodosa*. A positive correlation between temperature and shoot density, phenological parameters (number of leaves, leaf length, leaf width and biomass) and leaf elongation rates of *C. nodosa* has been found by other authors (Pérez and Romero 1992; Marbà et al. 1996; Cancemi et al. 2002; Tsioli et al. 2019). As already observed in other Mediterranean coasts, *C. nodosa* shows strong seasonal patterns in shoot density and phenological parameters, reaching the highest values in summer (Pérez and Romero 1992; Cancemi et al. 2002; Tsioli et al. 2019). The interactions showed that the strongest relationship is the one with *H. stipulacea*; in fact all interactions with it (*H. stipulacea* density-temperature, *H. stipulacea* density-salinity and third level interaction) are all negative. The temperature seems to have a buffering effect on the negative effect of *H. stipulacea* density, in particular the limiting effect is much stronger in high temperature conditions. We hypothesized that the limiting effect is due to the dense multi-layered mat formed by *H. stipulacea* rhizomes plus sediment, which push down *C. nodosa* rhizomes in a layer with oxygen depletion

(van Tussenbroek et al. 2016). Higher temperatures will cause an increase in oxygen depletion. The behavior of *H. stipulacea* seems comparable to that of invasive *Caulerpa* spp., able to negatively affect seagrass growth through the modification and deterioration of sediments (Borum et al. 2004; Holmer et al. 2009). According to Ceccherelli and Campo (2002), *C. cylindracea* would affect *C. nodosa* shoot density, but no clear evidence of a negative interaction between these two macrophytes was found.

The leaf features of the studied *H. stipulacea* population were generally comparable to those of other Mediterranean populations (Table 5). Moreover, we observed that the dimensions of the leaves of *H. stipulacea* do not seem to strictly depend either on the measured environmental variables or on the densities of the two species. In fact, they seem to depend on the categorical variable "Time", reflecting a temporal trend. On the other hand, shoot density of *H. stipulacea* presented some differences with respect to other Mediterranean populations (Table 5). The decline of *H. stipulacea* in January 2011 and the subsequent disappearance in April, could be related to changes in the environmental conditions that have become unfavorable (e.g. hydrodynamics, turbidity). Moreover, the occurrence in January of *C. cylindracea*, which rapidly expanded, invading the bare substrate left by *H. stipulacea*, may have prevented the re-establishment of *H. stipulacea*. Negative interactions and competitiveness between *Caulerpa* sp. and *H. stipulacea* have been already observed (Di Martino et al. 2006; Gab-Alla 2007).

Fertile plants of *H. stipulacea* were not observed in the study area. We know that they are much less common in the Mediterranean Sea than in the native habitat, suggesting a difficulty in completing sexual reproduction under the Mediterranean environmental conditions. Male flowers were mainly recorded in the Western Mediterranean (Cancemi et al. 1994; Procaccini et al. 1999; Gambi et al. 2009, 2018; Di Genio et al. 2021), suggesting that female flowers are not able to develop under the Western Mediterranean environmental conditions (Gambi et al. 2009). The recent finding of fertile plants bearing fruits on Chios Island and on Turkey coasts (Gerakaris and Tsiamis 2015; Dural et al. 2020), and flowers in Cyprus (Nguyen et al. 2018), indicates that in the Eastern Mediterranean *H. stipulacea* is able to reproduce sexually. Instead, the recent record of fruits in the Caribbean Sea (Chiquillo et al. 2019) has been questioned by Smulders et al. (2020), who believe that they likely are male flower buds, which have similar dimensions to fruits. The authors stated that the existence of female flowers and fruits of *H. stipulacea* in the Caribbean Sea is still an open question and confirmed that *H. stipulacea* reproduces only asexually in the Caribbean.

Since sexual reproduction has rarely been reported in invaded areas (Mediterranean and Caribbean Sea), the dominant way of dissemination and expansion seems to be vegetative propagation (Boudouresque and Verlaque 2002; Missaoui et al. 2003; Sghaier et al. 2011; Nguyen et al. 2018). Understanding reproduction of *H. stipulacea* in its invasive range is critical to managing this species. Indeed, sexual reproduction may increase the dispersal capacity and the adaptive capacity of this species and, through the seed banks, may increase its resilience to disturbances (Ackerman 2006; Unsworth et al. 2015; Smulders et al. 2020).

Although *H. stipulacea* highlights a relatively limited invasion success in the Mediterranean Sea if compared with the successful invasion reported for the Caribbean (see Winters et al. 2020) and has never shown any clear and regular invasive behavior (Di Martino et al. 2006; Gambi et al. 2009; Sghaier et al. 2011), it shows the potential for long distance dispersal (Willette and Ambrose 2009; Short et al. 2010) and possesses some features that could make it a potential threat to native seagrasses. Under warming scenarios, *H. stipulacea* could occupy the niche left by *P. oceanica* together with *C. nodosa*, continuing to support carbon sequestration, thus contributing in the mitigation of the global warming (Wesselmann et al. 2021). However, even though *H. stipulacea* might potentially contribute to increasing the carbon sequestration, the mainly allochthonous origin of organic carbon deposited in *H. stipulacea* sediments make it more susceptible to remineralization, implying a deterioration in the quality and quantity of the carbon. Furthermore, the weak rhizome structure of the species enhances the probability of sediment erosion and subsequent loss of sedimentary carbon stock (Apostolaki et al. 2019).

Conclusion

Certainly, our results represent a starting point and further investigation on the ecology and dynamics of *H. stipulacea* and its interaction with native seagrasses is needed. Indeed, in recent years, seagrass ecosystems have been experiencing a well-documented decline in many areas of the world (Boudouresque et al. 2009; Marbà and Duarte 2010; Marbà et al. 2014; Chefaoui et al. 2018), thus their conservation is becoming increasingly important (Unsworth et al. 2019). Under the climate change scenarios, the exotic *H. stipulacea* and the native warm tolerant species *C. nodosa* could substitute *P. oceanica* or even *H. stipulacea* could significantly outcompete the *Cymodocea* native species (Wesselmann et al. 2020). Genomic can be an important tool to better understand the ability of *H. stipulacea* to adapt to environmental conditions and spreading, and to respond to expected climate change (Specchia et al. 2017; Tsakogiannis et al. 2020; Winters et al. 2020; Zangaro et al. 2021).

CRediT author statement

Anna Maria Mannino: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing - Original draft, Writing - Review and Editing, Visualization, Supervision. Paolo Balistreri: Writing - Review & Editing. Francesco Paolo Mancuso: Data curation, Formal analysis, Writing - Review & Editing. Fabio Bozzeda: Data curation, Formal analysis, Writing - Review & Editing. Maurizio Pinna: Conceptualization, Data curation, Formal analysis, Writing- Review & Editing.

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RESEARCH ARTICLE

Temporal trends in the accumulation of alien vascular plant species through intentional and unintentional introductions in Japan

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Abstract

Clarifying the temporal trends of alien plant accumulation is increasingly important for informing global and national management efforts to decelerate biological invasions, following the adoption of Target 6 of the Kunming-Montreal Global Biodiversity Framework. However, such trends have not yet been analysed in many countries including Japan, which has the highest number of naturalised alien plant species among islands. To clarify the past and recent trends in the accumulation of alien plant species in Japan, we compiled a dataset of the year of first record for 1,463 alien vascular plant species deliberately and accidentally introduced and analysed the changes in the annual number of first records over time for each overall, intentional and unintentional introductions. We found that, overall, the annual number of first records of alien plant species in Japan began to increase in the late 1800s, and the increase continued until the late 1950s, with an estimated maximum of 15.7 new species per year. The increase then halted by 1960 and began a slow decline; the estimated average records per year between 1991 and 2000 dropped to 13.3 species. Since 1900, the annual number of first records associated with intentional introductions has more than doubled the number linked to unintentional introductions. Additionally, the proportion of invasive species brought through intentional introductions was larger than that brought through unintentional introductions. We highlight that while Japan experienced a rapid accumulation of alien plant species, including invasive species, by the 1950s, the rate of accumulation showed signs of saturation by 1960 and has since been slowly declining. Further deceleration and prevention of the introduction of invasive alien species, as targeted in the Kunming-Montreal Global Biodiversity Framework, may be achieved through increased investment in pathway management, especially management of intentional pathways.

Introduction

The number of alien species established outside their native range and the associated impacts on biodiversity and human wellbeing have explosively increased in recent centuries due to the acceleration of international trade and travel (Perrings et al. 2005; Westphal et al. 2008; Hulme 2009, 2021; Pyšek et al. 2010; Bonnamour et al. 2021). To avoid further negative impacts, slowing the rate of biological invasions is a pressing global matter (McGeoch et al. 2010; Hulme 2015), as set out in Target 6 of the Kunming-Montreal Global Biodiversity Framework (CBD 2022). Among alien taxonomic groups, vascular plants, which have long histories of utilisation, are one of the largest taxa that have been introduced beyond borders (van Kleunen et al. 2015). In addition to being a threat in itself, the introduction of alien vascular plants has led to additional invasions, because plants are vectors of other non-native organisms such as insects and pathogens (Sikes et al. 2018; Turner et al. 2021). Hence, information on the past and current rates of alien plant accumulation is particularly important for the development of management plans to slow the pace of biological invasions. To date, temporal trends in alien plant accumulation have been estimated at a global scale and at a national scale for several countries in Europe, North America and Oceania (Seebens et al. 2017, 2018) as well as for China (Ni and Deane 2022), and it has been shown that while the global accumulation rate has remained high since the 20th century, national trends have varied, with some countries showing a decline in accumulation rates in recent years (e.g., New Zealand). However, for many other countries, such trends remain unclarified, and it is unknown whether the accumulation of alien plants is continuing or saturated.

Japan is one of the island countries that has the largest number of naturalised alien vascular plant species (Pyšek et al. 2017); nonetheless, there is a lack of analysis of the temporal trends and major pathways of alien plant accumulation. Given that international trade was largely restricted in Japan until 1854 due to a national isolation policy (Asada 2000), the introduction and establishment of alien plants in the country must have proceeded over the following 150 years at a drastic pace involving various intentional and unintentional pathways. Previous studies have documented that intentional introductions, especially those for ornamental purposes, have generally contributed the most to alien plant invasion (Reichard and White 2001; Kowarik 2005). In fact, a global analysis has demonstrated that the majority of the world's naturalised alien species were introduced for ornamental cultivation (van Kleunen et al. 2018). The accumulation of alien plant species in Japan might also be substantially attributable to deliberate, ornamental introduction among various pathways, but this possibility has not yet been verified. Estimating the pace of accumulation of alien plant species in Japan, with its history of national isolation, and identifying the introduction pathways

that have contributed the most to this accumulation will enhance our understanding of global and regional mechanisms of alien plant invasions. Furthermore, doing so will provide an important basis for setting national management goals and targets to decelerate the introduction and establishment of invasive alien species, in line with the Kunming-Montreal Global Biodiversity Framework.

To achieve these goals, in this study, we compiled a dataset of the first record year and introduction pathways for approximately 1,500 alien vascular plant species found in Japan and analysed the changes over time in the annual number of newly recorded species (i.e., first record rates) by pathway throughout the past 150 years. We also calculated the fraction of invasive species brought through each pathway to identify the routes that have contributed the most to the accumulation of problematic alien species with detrimental impacts on biodiversity and human wellbeing. Here, we specifically determine (1) the past and recent trends in first record rates (accelerating, decelerating or saturated) and the period with the greatest first record rates and (2) the pathways through which more invasive species have been introduced and that should be targeted in future management.

Methods

Background information on Japan and its international trade

Japan is an island nation located in East Asia. Historically, the country conducted trade internationally, mainly with neighbouring Asian countries, and with a few European countries, until it was interrupted by the Edo shogunate, the first united government established in 1603 (Asada 2000). The government started prohibiting all international trade except for that with China and the Netherlands in 1639, mainly for religious control. This national isolation lasted over 200 years but officially ended in 1854, when the Japan–US Treaty of Peace and Amity (known as the Convention of Kanagawa) was signed between the United States and the Edo shogunate (Asada 2000). International trade has drastically developed, and Japan's trade partners have diversified since then (Yamazawa and Yamamoto 1979), creating more opportunities for biological invasions. Japan experienced dramatic economic growth after World War II ended in 1945, and international trade further expanded (Trade Statistics of Japan, https://www.customs.go.jp/toukei/suii/html/nenbet.htm). Complied long-term trade data show that the annual import value in 1950 was 15 times that in 1940, and this value has continuously increased since then to the present (Suppl. material 1).

Compilation of data on alien plant species

We generated a dataset on alien vascular plant species introduced from overseas to Japan using the following four steps. First, we created a list of alien plant species based on Yonekura (2012), a comprehensive plant inventory that enumerates the standardised scientific names and Japanese names of approximately 11,000 alien and native vascular

plant species recorded in the Japanese archipelago. From Yonekura (2012), we extracted all alien species, infraspecific taxa such as subspecies and varieties, and hybrids (hereafter referred to as species for simplicity) that have naturalised (i.e., maintained self-sustaining populations) and those that have not yet been confirmed to have naturalised but are frequently observed to escape cultivation. We restricted our listing to alien species that the literature clearly identified as naturalised or escaped, and we excluded species described as "possibly" naturalised or escaped to ensure data quality. The resulting list included a total of 1,753 species. Although the species names extracted from Yonekura (2012) for our list did not entirely correspond to the names accepted in international taxonomic sources such as The WFO Plant List (https://wfoplantlist.org/plant-list/), they were considered widely referenced in Japan. Therefore, we used the species names in the list (for taxa below the species level, those including infraspecific epithets) as a standard against which to standardise species names reported in other information sources. The standardisation was conducted referring to Ylist (http://ylist.info/index. html), a web-based nomenclatural index of names of vascular plants in Japan, which was created by the same author as Yonekura (2012) and follows the same taxonomy.

Second, we gathered information on the year of first record of each listed species from four comprehensive illustrated reference books on alien or cultivated plant species in Japan: Honda et al. (1988a, b, c, d), Shimizu et al. (2001), Shimizu (2003) and Uemura et al. (2010). If the year of first record was shown as a period such as the "1950s" or "Meiji era", the middle year of the term was employed. Similarly, if the year was provided as the "early", "late" or "end" of a certain period, we applied "first year of the period + 1", "the middle + 1" or "the last year - 1", respectively (for example, 1991 for "early 1990s"). In cases where there was an inconsistency in the years among the literature, the oldest was employed. Based on the four reference books, we identified the year of first record for 48% of species on the list. To fill the data gap, we further referred to the Science Museum Net (https://science-net.kahaku.go.jp/, accessed in June–August 2022), a database of specimen records kept in more than 80 natural history museums across Japan (Hosoya et al. 2018). From the database, we extracted the collection year of the first (i.e., oldest) specimen of the species, which allowed us to increase the proportion of listed species with the year of first record to 86%. We also collected information on the year of introduction, where possible, from the four illustrated books described above and an encyclopaedia on the history of plant cultivation in Japan (Shimizu 1984) to identify species that were introduced before 1603, when the Edo shogunate was established and literacy rates began to increase (Saito 2012). The reason is that it is highly likely that those archaeophyte species were discovered but not recorded in written form before the year of first record collected from the books or specimens and therefore should subsequently be excluded from analysis. Information on the year of introduction was used only to identify archaeophyte species and was not incorporated into the analysis.

Third, we compiled information on the introduction pathways of the listed species or descriptions of how the species was used as a surrogate for the introduction pathway. We applied a major pathway classification, i.e., intentional and unintentional. We further classified them into pathway categories defined by the Convention on Biological

Diversity (CBD 2014) but only employed release in nature and escape from confinement as intentional pathway categories and contaminant and stowaway as unintentional pathway categories. Corridors and unaided spread categories were not relevant, considering the geographical conditions of Japan as an island nation. As the number of species associated with escape from confinement was substantially large, we subdivided this category into six subcategories, taking into account the conditions of plant cultivation in Japan (Suppl. material 2), i.e., escape from agriculture, botanical gardens, food for pets, medicinal and industrial purposes, ornamental purposes and research, which are partially compliant with the CBD subcategories detailed in Harrower et al. (2020). As the source of information on introduction pathways, we first consulted the four illustrated reference books described above and then further referred to most of the available literature specifically including a list of unintentionally introduced alien species in Japan (Japan Forage Crop Seeds Association 1972; Murayama et al. 1989a, b; Asai et al. 2007, 2009; Shimono and Konuma 2008; Ikeda et al. 2022). The reason is that unlike intentional introductions, unintentional introductions are often not recorded (Lehan et al. 2013) and, as a result, may be incompletely presented in the illustrated reference books. For each species, we recorded all pathways for which evidence was available, not only one main pathway. The species for which no information on introduction pathways was found in any of the literature were grouped as species of unknown pathways.

Finally, we compiled information on whether each species has been recognised by the Japanese government as harmful to biodiversity and human wellbeing by referring to two invasive alien species lists created by government agencies, one made in 2005 ("the alien species alert list", Ministry of the Environment 2005) and the other made in 2015 ("the list of alien species that may have adverse effects on ecosystems in Japan", Ministry of the Environment and Ministry of Agriculture, Forestry and Fisheries 2015). The alien species on the lists were selected based on their impacts on native species, ecosystems, human health and economic activities (Ohsawa and Osawa 2014; Egawa and Matsuhashi 2022). Although these lists are not legally binding in themselves, they have been developed to identify and raise public awareness of harmful species that need to be managed. In the present study, we defined invasive alien species as species included on one or both of the government lists, i.e., species that have serious impacts and a recognised need for management at a national level.

The compiled dataset included 46 archaeophyte species that were introduced before 1603, and we removed those species to ensure data quality. Doing so resulted in 1,707 species left for analysis, of which 1,463 species had information on the year of first record.

Data availability

The dataset generated during this study is considered the largest of first records for alien vascular plant species in Japan, given that the number of alien vascular plant species tagged with Japan currently registered in the Alien Species First Records Database (Seebens 2021) is 165. The dataset is available at https://doi.org/10.5281/zenodo.7597598 (Egawa and Koyama 2023).

Data analysis

All data analyses were performed using the R statistical environment (R Core Team 2020). The cumulative number of first records throughout was calculated for each overall, pathway category and subcategory, using data on 1,463 species with information on the year of first record. First record rates (i.e., annual number of species newly recorded) were also calculated for overall and each of the same pathway groups as above. To check the association between the first record rate and trade value, the correlation between the overall first record rate and annual import value was tested using Spearman's rank correlation coefficients (Suppl. material 1).

The temporal trends of the first record rates were modelled using generalised additive models (GAMs) in the mgcv R package. The GAMs enabled us to illustrate nonlinear trends over time. Because of the nature of first record rates as overdispersed count data, we applied a negative binomial distribution with a log link function. In addition to the overall trend without distinguishing introduction pathways, a separate model was constructed for each pathway category and subcategory having a sufficient sample size, which is here defined as more than 40 species. The theta parameters of negative binomial distributions were estimated during model fitting. If the relationship between time and the first record rates was not significant at $p < 0.05$ for a given pathway according to the GAM, we considered that the first record rates associated with the pathway did not change temporally. In our dataset, the first records of alien plant species before 1845 were found in only three fragmentary years (1699, 1735 and 1784), and the number of recorded species in these three years was limited to five. Likewise, first records after 2000 (110 species) were possibly underrepresented due to the delay between observation and report, as seen in the sharp decline in the number of first records in the last 20 years (Suppl. material 1: fig. S1-2B). Therefore, we restricted our GAM analysis to 1,348 species for which the first record years were identified between 1845 and 2000.

To determine the pathways responsible for the accumulation of invasive alien species, we tested whether the proportion of invasive species to all species introduced through each pathway differed from the overall pattern without distinguishing pathways using Fisher's exact test with Bonferroni correction of p values in the RVAideMomoire R package. We considered pathways with a significantly higher proportion of invasive species than the overall pattern as more responsible in terms of invasive species introduction. The analysis was conducted only for pathways involving more than 40 species.

Results

Temporal changes in cumulative number and first record rate of alien plant species

The cumulative number of first records of alien plant species in Japan has been increasing since the late 1800s due to both intentional and unintentional introductions and largely due to introduction through unknown pathways (Fig. 1A). Among the

intentional pathways, escape from ornamental uses contributed the most to the accumulation of new species, followed by escape from agriculture, throughout the investigation period (Fig. 1B). For unintentional pathways, the contribution of contaminants was far greater than that of stowaways throughout the investigation period (Fig. 1C).

The GAM analysis detected significant temporal trends in first record rates during the period from 1845 to 2000 for overall and all the examined pathways except for release in nature (Fig. 2, Suppl. materials 3, 4). The overall first record rate of alien plant species in Japan began to accelerate in the late 1800s and continued until the late 1950s, with an estimated maximum of 15.7 new species per year during the period from 1955 to 1960 (Fig. 2A). Since 1961, the first record rate has slowly decelerated (Fig. 2A); the model-estimated average annual first record rate during the recent 10-year period from 1991 to 2000 was 13.3 species. Spearman's correlation coefficients showed that the overall first record rates had been significantly associated with the annual import value approximately 10 years earlier until 1950, but the association with the import value disappeared after 1960 (Suppl. material 1). The GAM-estimated temporal trends of first record rates of intentional and unintentional introductions were also of a one-peak type, although the timing of the peak differed between pathways. The first record rate associated with intentional introduction peaked at 5.6 new species per year around 1940 and started to decelerate from 1946, averaging 3.2 species per year in recent years from 1991 to 2000 (Fig. 2B). This pattern was largely attributable to the patterns of two main intentional pathways, escape from ornamental uses, with a maximum of 3.7 species per year in the 1940–1947 period, and agriculture, with a maximum of 1.5 species per year in the 1931–1942 period (Suppl. material 4). The first record rate associated with unintentional introduction, which was mostly attributable to contaminants, peaked around 1945, with an estimated maximum of 2.4 new species per year, and started slowing around 1950 (Fig. 2C). The average first record rate through unintentional introduction in the recent 10-year period was estimated at 1.4 species per year. Compared to intentional and unintentional introductions, the first record rate relevant to unknown introduction peaked later, i.e., around 1965, with an estimated maximum of 9.4 species per year. The rate remained largely unchanged until 2000 (Fig. 2D).

The proportion of invasive species by pathway

The proportion of invasive species to all species varied across introduction pathways (Fig. 3). Compared to the overall pattern not distinguishing pathways (11%, 193 in 1,707 species), the proportion of invasive species brought through intentional introductions was significantly higher (20%, 109 in 549 species; $p < 0.001$, Fisher's exact test with Bonferroni correction). Three intentional pathways, i.e., escape from ornamental and agricultural purposes and release in nature, showed significantly higher proportions of invasive species than the overall pattern (Fig. 3; $p < 0.05$). In contrast, the proportion of invasive species brought through unknown pathways was significantly lower (6%, 65 in 1,022 species; $p < 0.001$) than the overall proportion. The proportion of invasive species introduced through unintentional pathways (18%, 40 in 220 species) did not statistically differ from the overall pattern (Fig. 3; $p = 0.205$).
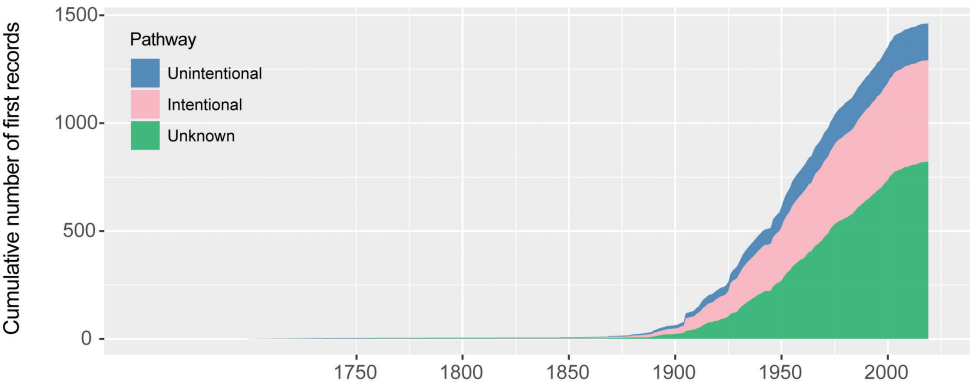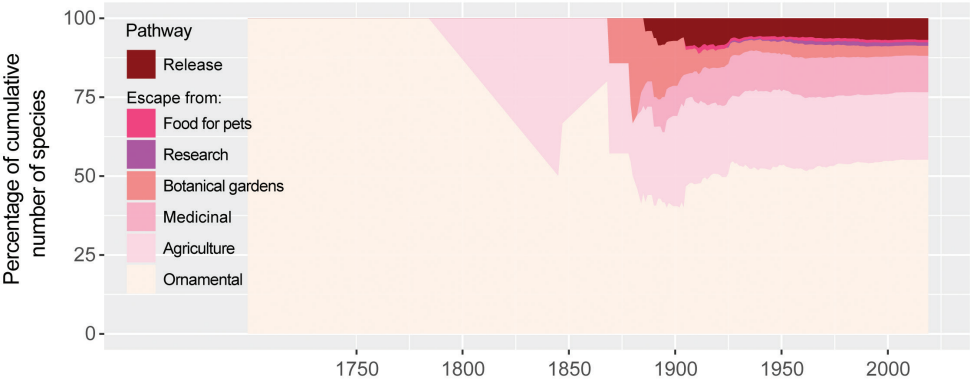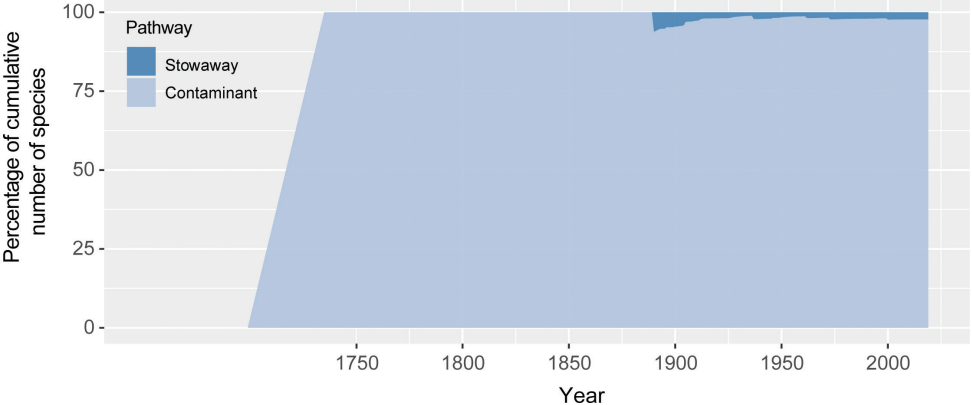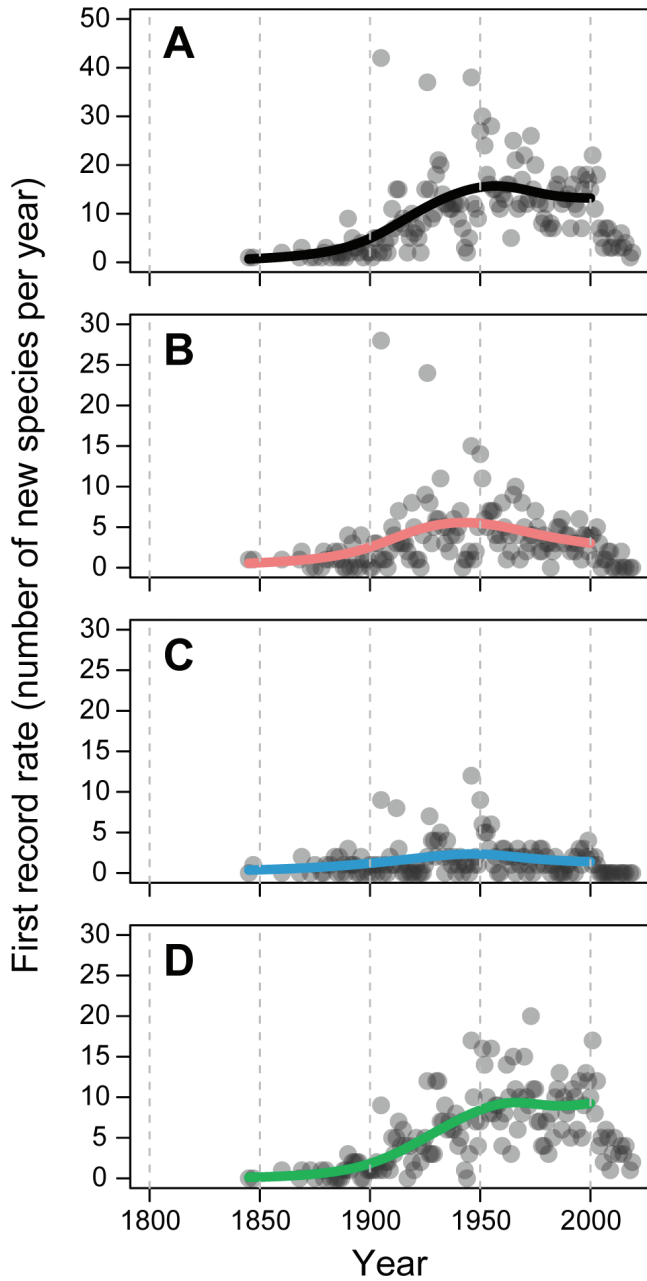
Figure 1. Cumulative number of alien plant species by introduction pathway in Japan **A** cumulative number of first records **B, C** percentage of species introduced by each pathway in the cumulative number of species introduced via intentional and unintentional introductions, respectively.

Figure 2. Temporal trends in first record rates in Japan, 1845–2000 **A** overall trends without distinguishing pathways **B–D** trends for intentional, unintentional and unknown introductions, respectively. Grey dots indicate the observed numbers of first recorded species, and lines indicate the generalised additive model (GAM) estimations (for the model fitting results, see Suppl. material 3). Note that the observed numbers of the first recorded species after 2000 are shown for reference purposes but were not included in the GAM analysis.
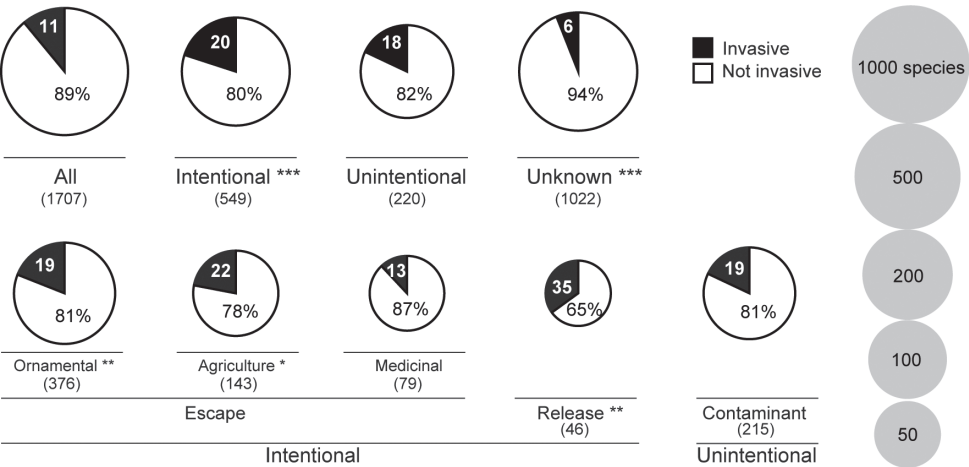
Figure 3. Proportion of invasive species by pathway with more than 40 species involved. Asterisks indicate significant differences in the proportion of invasive species compared to the overall pattern (All) without distinguishing pathways (Fisher's exact test with Bonferroni correction of p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The numbers in brackets and size of the pie charts show the total number of species involved in the pathways. As one species may be included in multiple pathways, the number for each subcategory amounts to more than the overall number (All).

Discussion

Our analysis using a newly compiled dataset yielded the following two key findings on the temporal trends and main pathways of alien plant accumulation in Japan: (1) starting in the late 1800s when the country opened its borders, the accumulation of alien plant species accelerated, but by 1960, the pace of accumulation showed signs of saturation and has been slightly decreasing since then to the present. (2) The accumulation of invasive alien species with detrimental impacts on biodiversity and human well-being is largely attributed to intentional introductions, including those for ornamental purposes, as well as agricultural purposes and release in nature.

Our estimation of the maximum overall first record rate in Japan, 15.7 new alien plant species per year, was 1.8 times the maximum rate of 8.8 species per year recorded in China in the early 1900s (Ni and Deane 2022). Similarly, the maximum first record rate that we obtained was greater than the peak rates estimated in a previous study on other islands, such as the United Kingdom and the Hawaiian Islands (Seebens et al. 2017). Given that we collected the year of first record for only 86% of the species in the compiled list, the actual first record rates in Japan should be even greater than the estimation shown here. Such an intensive accumulation of new species would have led to the largest number of naturalised alien plant species in Japan among island nations.

However, we found that the overall first record rate peaked in 1955 and slowly began to decline in 1961. This saturation and subsequent decline are possibly due to multiple social and environmental factors. For example, the reduced access to new alien species pools because of the saturation of the diversity of trade partners can lead

to a slowdown of the first record rate (Seebens et al. 2015, 2018). This process may be relevant to Japan because although Japan's import partners diversified immediately after the opening of the country, the North American share stayed high, especially after World War II ended in 1945 (Yamazawa and Yamamoto 1979). Another possible explanation is increased biosecurity efforts. Japan enacted the Plant Protection Act in 1950 and enforced a systematic import quarantine of vascular plant species that may carry plant pests and diseases (Sakata 2011). Additionally, since 1967, Japan has participated in the Organisation for Economic Co-operation and Development (OECD) Schemes for the Varietal Certification of Seed to ensure the use of high-quality agricultural seeds with reduced contamination risk. These import-related biosecurity efforts may have contributed to the recent slowdown of the first record rate since 1961. These explanations are also consistent with the results of correlation analysis showing that after 1960, the association between the first record rate and import value disappeared, with the annual number of newly recorded species not increasing even as import value increased (Suppl. material 1). In addition to these factors, temporal changes in land use and environmental suitability due to climate change might be involved in the saturation of first record rates, as suggested by previous studies (Walther et al. 2009; Seebens et al. 2021). All factors are not mutually exclusive and could thus have contributed interactively.

As above, we found a promising tendency in which, overall, the accumulation of new alien plant species in Japan has been saturated and even slowing despite the continuing trade expansion. Nonetheless, the estimated first record rate in the recent 10-year period from 1991 to 2000 was still more than 13 species per year. Based on the proportion of invasive species to all introduced species (11%), the first record rate of 13 new species per year implies that at least one novel invasive species is included every year. We found that the first record rate via intentional introduction exceeded the rate via unintentional introduction throughout the study period. This finding is in line with previous studies highlighting the primary roles of intentional introduction in alien plant invasion (Lonsdale 1994; Hulme et al. 2008). In particular, escape from ornamental horticulture is known as the major introduction pathway for non-native species in Europe and regions with cultural legacies of European colonialism (Lehan et al. 2013; van Kleunen et al. 2018). Our results showing the importance of intentional introduction, especially for ornamental purposes, in the accumulation of new species are in line with this pattern, although Japan historically has a different cultural sphere from Europe. We also found that intentional introductions are even responsible for the introduction of invasive species, specifically defined here as species on the government's alert lists for their detrimental impacts on biodiversity and human wellbeing. Similarly, in China, 50% of invasive alien plant species were intentionally introduced for various purposes (Xu et al. 2006). These results suggest that, in line with the global direction (Perrings et al. 2005), managing intentional introductions is key to reducing the accumulation of invasive alien plant species in East Asia. In the present study, information on specific introduction pathways was not found for 60% of all listed species. We assume that the majority of the species with unknown pathways were unintentionally

introduced as contaminants or stowaways because intentional introductions of species are more likely to be recorded (Lehan et al. 2013). Therefore, in this study, species associated with unintentional introductions are presumably underrepresented. However, the possible underestimation of unintentionally introduced species does not affect the implication of the study that the management of intentional introduction holds particular importance for slowing the accumulation of invasive species in Japan. The reason is that the proportion of invasive species brought through unknown pathways was much smaller than that brought via other pathways. Hence, combining data on species with unknown pathways does not increase the fraction of invasive species brought through unintentional introduction.

Conclusion

This study demonstrated the long-term trend of alien vascular plant accumulation in Japan and the introduction pathways that have most contributed to the accumulation. We believe that the results of this study could be a basis for developing national policies and action plans to achieve Target 6 of the Kunming-Montreal Global Biodiversity Framework, which aims to reduce the rates of introduction and establishment of invasive alien species by at least 50% (CBD 2022). If the first record rate of alien vascular plants was to be reduced by half in Japan, based on our estimation of the recent 10-year period from 1991 to 2000, the rate should be cut to at least 7 new species per year. Further investments and efforts in pathway management, especially the management of intentional pathways, will be needed to achieve this rate, but they will ultimately contribute to eliminating the impacts of invasive alien species and conserving biodiversity and human wellbeing.

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Supplementary material 1

Association between the first record rate and import value

Authors: Chika Egawa, Asuka Koyama

Data type: text including figures and a table (PDF file)

Explanation note: Methods and results of testing the association between the first record rate and import value.

Link: https://doi.org/10.3897/neobiota.83.101416.suppl1

Supplementary material 2

Definitions of subcategories used for escape from confinement

Authors: Chika Egawa, Asuka Koyama

Data type: table (PDF file)

Explanation note: Definitions of subcategories used for escape from confinement in this study.

Link: https://doi.org/10.3897/neobiota.83.101416.suppl2

Supplementary material 3

Generalised additive model (GAM) results

Authors: Chika Egawa, Asuka Koyama

Data type: table (PDF file)

Explanation note: Generalised additive model (GAM) results of the temporal trends in the first record rates.

Link: https://doi.org/10.3897/neobiota.83.101416.suppl3

Supplementary material 4

Temporal trends in first record rates from 1845 to 2000 by subcategory of introduction pathway

Authors: Chika Egawa, Asuka Koyama

Data type: figure (PDF file)

Explanation note: Temporal trends in first record rates from 1845 to 2000 by subcategory of introduction pathway.

Link: https://doi.org/10.3897/neobiota.83.101416.suppl4