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A perennial invader's seed and rhizome differ in cold tolerance and apparent local adaptation

Vasiliy T. Lakoba[1], Gregory E. Welbaum[2], John R. Seiler[3], Jacob N. Barney[1]

1 *School of Plant and Environmental Sciences, Virginia Tech, 675 Old Glade Road, Blacksburg, Virginia, USA*
2 *School of Plant and Environmental Sciences, Virginia Tech, 1880 Pratt Drive, Blacksburg, Virginia, USA*
3 *Department of Forest Resources and Environmental Conservation, Virginia Tech, 310 West Campus Drive, Blacksburg, Virginia, USA*

Corresponding author: Vasiliy T. Lakoba (vtlakoba@vt.edu)

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Abstract

Extreme cold plays a key role in the range boundaries of plants. Winter survival is central to their persistence, but not all structures are equally susceptible to frost kill and, therefore, limiting to distributions. Furthermore, we expect intraspecific variation in cold tolerance both within and among tissue types. In a laboratory setting, we determined freezing tolerances of two overwintering propagule types – seeds and rhizomes – of the globally invasive Johnsongrass (*Sorghum halepense*), testing apparent emergence and electrolyte leakage as a proxy for cell death. We used 18 genotypes from agricultural and non-agricultural habitats spanning the climatic extremes occupied by Johnsongrass in the US. Single node rhizome fragments had an average LT90 of -5.1 °C with no significant variation based on home climate or ecotype. Seeds frozen at -85 °C suffered a decline in germinability to 10% from 25% at 22 °C. Population origin did not affect seed response to any temperature. However, non-agricultural seeds germinated more and faster than agricultural seeds from the coldest climates, with a reversed relationship among warmest origin seeds. Regardless of ecotype, seeds from the cold/dry and wet/warm sectors of Johnsongrass's range germinated more and faster. Drastic differences in cold tolerance between seeds and rhizome and evidence for seeds' local adaptation to land use and climate suggest that its spread is likely limited by winter rhizome survival, as well as adaptability of germination behavior to longer winters. These findings shed light on Johnsongrass' dispersal dynamics and help identify future avenues for mechanistically understanding its range limitation.

Keywords

Cold tolerance, invasive plants, land use change, local adaptation, range boundaries

Introduction

Species range limits are often dictated by climatic tolerances at large spatial scales. For most plants, temperature and moisture availability play a leading, though not unilateral, role in defining distributions (Curtis and Bradley 2016). To characterize how specific temperature or moisture parameters (e.g., extremes, durations, seasonality) limit a plant's range, empirical methods and spatial models are typically used (Kotta et al. 2019; Greiser et al. 2020). In the temperate biomes of the northern hemisphere, cold winter temperatures play a role in limiting some perennial plants' northern range boundaries, but are not sole drivers, interacting with growing season conditions that shape overwintering tissue maturation (Körner et al. 2016). While winter survival is measured at the whole-plant level, it is important to consider that a variety of organs (e.g., roots, herbaceous or woody shoots) may be responsible for cold tolerance to different degrees (Washburn et al. 2013; Sage et al. 2015; Ambroise et al. 2020). This means that distribution modeling based only on species presence is not sufficient to mechanistically identify cold tolerance limits (Gardner et al. 2019), especially in herbaceous perennials which often look similar in each season of their lifespan. Specifically, summer presence of such a species does not necessarily indicate survival through the previous winter, but potentially successful re-establishment from seed. This, in turn, muddles our understanding of how the plant and its constituent tissues respond to freezing temperatures. Cold tolerance is often considered at the organismal level, but testing different organs separately is most appropriate, particularly for perennial plants.

Both sexual and vegetative structures need cold tolerance to survive between growing seasons in non-tropical climates. While individual perennation is dependent on winter cold tolerance (Sage et al. 2015), sexual reproduction is dependent on annual seed production, dispersal, and establishment. In many temperate species, the winter season also intercedes between seed abscission and germination, requiring seed cold tolerance (Leiblein-Wild et al. 2014). Given the importance of cold tolerance to both survival and reproduction – and, therefore, overall fitness – its parameters (e.g, lethal temperature estimates) can be projected onto spatial climate patterns to hypothesize, though not predict, range limits (Sánchez-Fernández et al. 2012; Gardner et al. 2019). The strength of this approach is not in accurately and causally predicting distribution (which is better served by correlative modeling of multiple drivers), but in understanding the locations at which the specific stressor (i.e., temperature) is or is not likely to limit distribution.

Perenniality is a boon to plant fitness because it reduces each subsequent year's demand for vegetative-to-reproductive allocation (Rohde and Bhalerao 2007). However, this requires major structural (e.g., xylogenesis) and non-structural carbohydrate production (Kozlowski 1992; Slewinski 2012), whose storage is mediated by winter minimum temperatures (Li et al. 2017). This is a key to some plants' persistence through seasonal energy reallocation and storage in rhizome tissue (Boström et al. 2013). Across climate gradients, conditions acting on perennial tissue survival select for locally hardy (i.e., cold tolerant) genotypes (Malyshev et al. 2014; Dong et al. 2019). This selection can be driven not only by macroclimates, but also by anthropogenically-induced microclimates (e.g., irrigated fields, urban heat islands, etc.) and resource subsidies (Charrier et al. 2015; Oraee et al. 2020).

Perenniality can also buffer against challenging growing conditions (e.g., cold winter temperatures, Wingler 2015), and therefore impact species distribution. An important link between perenniality, fitness, and distributions is dispersal capability, which we can better understand by comparing propagules of varying anatomy, stress tolerance, and transportability (e.g., seed vs. rhizome).Thus, if local climates and habitats (i.e., provenance) select for biologically significant differences in cold tolerance and perenniality, this raises two questions. Firstly, could ranges suitable for vegetative propagation be more provenance-limited than ranges suitable for seed propagation? Secondly, if habitat type impacts perenniality via cold tolerance, could species distribution be mediated by land use as a selection pressure? These two questions have not been explicitly studied, but we might anticipate many interactions between land management and adaptation in current and future climates (Ramesh et al. 2017; Weber et al. 2017).

Advantages of having rhizomes (perennial underground stems) are evident across many plant systems (e.g., Chen et al. 2015; Grewell et al. 2019). In particular, rhizomes are a common feature of invasive species, which lend themselves well to the study of range limits and stress tolerance due to high data availability and ease of propagation. For example, some perennial invaders are able to maintain their competitive edge in recipient communities despite initial reductions in growth due to rhizome fragmentation (Zhou et al. 2017). In other cases, perennial invasive plants emerging from rhizomes are more competitive and stress tolerant than conspecific seedlings (Mitskas et al. 2003; Acciaresi and Guiamet 2010). Other forms of modified perennial stems, such as stolons, are believed to buffer invasive plants from stress and promote colonial expansion (Roiloa and Retuerto 2016). For instance, Pompeiano et al. (2015) found metabolic adjustment of sugar and proline concentration across all organs of the rhizomatous invader *Arundo donax* to explain differences in cold hardiness between Hungarian and Honduran populations. Studying populations from across a climate gradient, Dietrich et al. (2018) found *Dactylis glomerata* rhizome cold tolerance to correlate negatively with mean precipitation at home habitats across Europe. However, it remains unclear which climate and habitat factors drive variation in rhizomatousness in perennial invasive plants, and whether these species may forgo perenniality to colonize more challenging ranges. To address this, we used a model perennial invasive grass to evaluate the effects of climate and habitat on both rhizome and seed cold tolerance.

The cosmopolitan invader Johnsongrass (*Sorghum halepense*) sexually reproduces annually through seed, while its perenniality is achieved by rhizome survival through the winter (Washburn et al. 2013). It has been estimated that a single Johnsongrass genet can produce 33,600 kg ha^{-1} of rhizome annually (McWhorter 1972). The per-propagule establishment efficiency of rhizome over seed is another factor in its importance to the invader's persistence (Atwater et al. 2017). At Johnsongrass's northern range edge in southern Ontario, there are reported persistent annual populations which are presumed to be caused by failure of winter rhizome survival (Warwick and Black 1983). Given the abundant evidence for great intraspecific variation in Johnsongrass

(Atwater et al. 2016, 2017; Sezen et al. 2016), it appears likely that large differences in minimum temperature across this plant's U.S. range could have led to local adaptation of rhizome cold tolerance based on climate.

Rhizome cold tolerance may also be related to overall rhizome development, which responds to resource inputs. Fertilization and irrigation can be responsible for rhizome development changes compared to growth in non-agricultural settings (Schmid and Bazzaz 1992; Schwinning et al. 2017), therefore selection may be different in cropland habitats where more rhizome biomass is generated than in more stress-associated non-agricultural environments. This advantage in growing season resources may buffer against winter kill, similar to the way that trees rely on tissue maturation to survive freezing temperatures (Körner et al. 2016).

Specific to Johnsongrass, Atwater et al. (2017) found that, while seed was more efficient at reproducing than rhizome on a per-unit-carbon basis, rhizome was more efficient than seed per propagule. The same study found Johnsongrass plants emerging from rhizome fragments to be more sensitive to habitat variation, competition, and density than seedlings. In addition, there has been abundant evidence of ecotypic differences interacting with home climate and response to competition in Johnsongrass in the United States (Atwater et al. 2016). These pieces of information suggest that the species' different reproductive allocation strategies could be mediated by habitat type. However, no studies had isolated winter rhizome survival – on which the invader's perenniality depends – as affected by home climate or habitat. Fletcher et al (unpublished data) found populations from across the U.S. range to be incapable of winter survival at the northern range edge (Ithaca NY) regardless of ecotype, as opposed to 100% survival in Virginia, Texas, and New Mexico. Home climate- and ecotype-based differences in Johnsongrass's perenniation have remained an important knowledge gap. Combining our awareness of photosynthetic differences between agricultural and non-agricultural populations (Kelly et al. 2020; Lakoba and Barney 2020) and tissue maturation's role in cold tolerance (Körner et al. 2016), we chose to test for ecotypic differences in seed and rhizome cold tolerance.

In the broader context of plant invasion biology, we set out to test whether adaptation to different land uses can yield divergent stress adaptation in a relatively short period of time (i.e., decades to centuries). While other studies have investigated differences between geographic ecotypes and home climates as predictors of perennial plant cold tolerance (Pompeiano et al. 2015; Dietrich et al. 2018), ours is the first to compare climate origin with land use origin. To address this research gap, we subjected populations of agricultural and non-agricultural Johnsongrass ecotypes representing a wide range of home climates to sub-zero temperatures to evaluate rhizome and seed cold tolerance. Specifically, we wanted to know whether: 1) populations from colder and/or drier climates exhibit greater cold tolerance; 2) populations from non-agricultural habitats exhibit greater cold tolerance due to the lack of agricultural inputs aiding growth and non-structural carbohydrate storage; 3) cold tolerance trends within and among populations differ between seeds and rhizomes. The findings will help us further understand the implications of habitat switching in Johnsongrass as well as offer a new link between land use change, climate, and invasive species.

Methods

Population selection

We sourced propagules from our collection of >200 Johnsongrass populations representing the full geographic and climatic variation of its US range. In particular, we drew from a subset of this collection that consisted of seed produced in a common garden setting to account for maternal effects. For this study, we systematically chose populations representing both agricultural and non-agricultural origins, as well as the extremes of mean annual precipitation (MAP) and minimum January temperature (MinT), each averaged across a 30-year span (1981–2010). We used MAP to account for general moisture availability, which interacts with temperature, but is not the focus of our stress tolerance study. However, we chose January MinT as a proxy for the extreme cold experienced at a given location, which may correspond more directly with adapted cold tolerance rather than the annual mean (Curtis and Bradley 2016; Bishop et al. 2017). MAP and MinT values for each population's geographic origin were obtained from PRISM Climate Group (Oregon State University) using ArcMap 10.5.1 (ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute). Agricultural and non-agricultural populations were plotted separately in climate space (MAP vs. MinT). Home habitat MAP values ranged from 195 mm to1480 mm for agricultural populations and 198 mm to 1540 mm for non-agricultural populations. Home habitat MinT values ranged from -7.9 °C to 5.2 °C for agricultural populations and -9.4 °C to 4.3 °C for non-agricultural populations. We chose 12 populations of agricultural and non-agricultural origin (24 total) from the periphery (extremes) of the MAP vs. MinT plot (see Fig. 1). Of these,

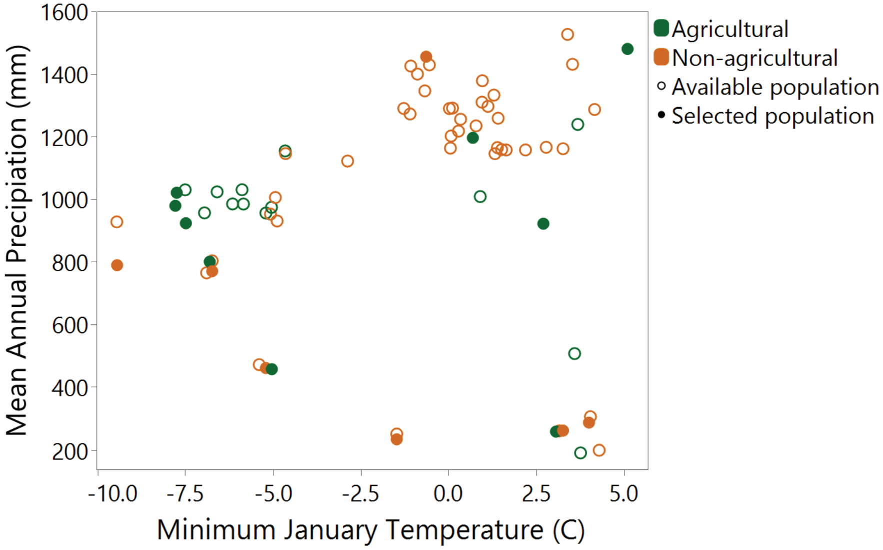

Figure 1. The Johnsongrass populations selected for the rhizome and seed experiments (see details in Table 1) plotted in climate space.

Table 1. A complete list of the Johnsongrass populations used in the seed freezing and rhizome freezing experiments. The ecotype source and the population is indicated, as well as the 30-year normal of mean annual precipitation (MAP) and minimum January temperature (MinT).

Population	Ecotype	MAP(mm)	MinT(°C)	Rhizome data	Seed data
CA-2	non-agricultural	262	3.26	yes	yes
TX-1	agricultural	923	2.7	yes	yes
NM-4	non-agricultural	234	-1.48	yes	no
AL-10	non-agricultural	1456	-0.64	yes	yes
GA-6	agricultural	1197	0.69	yes	yes
KS-4	agricultural	801	-6.81	yes	yes
TX-2	agricultural	1481	5.1	yes	yes
AZ-2	non-agricultural	306	4.0	yes	yes
FL-3	non-agricultural	1287	4.16	yes	no
AZ-3	non-agricultural	199	4.29	yes	yes
OH-7	agricultural	1022	-7.75	yes	no
KS-2	non-agricultural	771	-6.74	yes	yes
OH-1	agricultural	980	-7.79	yes	yes
NM-12	agricultural	458	-5.04	yes	yes
OH-8	agricultural	924	-7.49	yes	yes
NE-1	non-agricultural	790	-9.45	yes	yes
CA-1	agricultural	259	3.06	yes	yes
TX-4	non-agricultural	462	-5.21	yes	yes

11 populations from each ecotype (22 in total) successfully germinated and established seedlings in the greenhouse (see detailed description below). We then transplanted two seedlings of each population to a common garden on Virginia Tech's campus to generate rhizomes for the experiment. Following 6 months of growth in the common garden, nine populations from each ecotype (18 total) produced sufficient rhizome material for the rhizome freezing experiment. For the seed freezing experiment, we began with the 18 populations used in the rhizome experiment. Of these, 3 populations did not germinate under any conditions and were excluded from data analyses. Thus, we analyzed rhizome cold tolerance of 18 (9 agricultural, 9 non-agricultural) populations and seed cold tolerance of 15 (8 agricultural, 7 non-agricultural) populations (see Table 1).

Rhizome freezing experiment

To release seeds from dormancy, we treated them with commercial strength sodium hypochlorite (Clorox Regular-Bleach, The Clorox Company, Oakland CA) for 4 hours followed by a 1 hour water rinse (Atwater et al. 2016). The prepared seeds were then placed in Petri dishes (one dish per population) with four saturated sheets of filter paper (Whatman 1003-055, GE Healthcare, Chicago IL). Upon radicle emergence, seeds were transferred to 1800 cm^3 plastic pots filled with a potting mix (Miracle-Gro Moisture Control Potting Mix, Scotts Co LLC, Marysville OH) and allowed to establish for 4 weeks. All seedlings were then transplanted to 3.8 liter plastic pots (16.5 cm diam., 20 cm depth) filled with the same potting mix. After 4 months of growth in the greenhouse, two individuals from each population were then transplanted to a common garden dominated by fine-loamy Alfisols and Ultisols, where they were randomly distributed on a 6 m × 4.5 m grid. Plants were watered as necessary (once every ~14 days) and the ambient weed community was mechanically suppressed every 14 days as needed. Rhizomes were harvested from a single randomly-chosen individual from each population in December 2019.

Ten rhizome segments (10–20 mm long; containing only one node) from each plant (representing a single population) were sealed individually in capped 5 mL plastic culture tubes (Samco DCT, Thermo Fischer Scientific, Waltham MA) and submerged in cooling baths of 50:50 ethylene glycol:water solution for the cold treatments. We limited the rhizome segments to one node due to the known inverse relationship between segment length and probability of emergence in Johnsongrass (McWhorter 1972). Due to the number of cooling baths available, two populations – chosen at random from the common garden – were treated at one time for a total of four populations per day. This random selection of populations each day accounted for the five day spread in harvest times across all populations. Rhizome segment fresh biomass was recorded at this time for later use as a covariate to account for possible size differences. All treatments were administered for 6 hours, beginning at 5 °C for 1 hour and dropping to one of the following temperatures: 0 °C, -2 °C, -4 °C, -6 °C, or -10 °C for the remaining 5 hours. Acclimation periods – whether stepped or continuous – are standard protocol for tissue cold tolerance studies (Fiebelkorn and Rahman 2016; Peixoto and Sage 2016; Leuendorf et al. 2020), while the six-hour duration was chosen based on known lethality of -3 °C to -5 °C temperatures for Johnsongrass well within 24 hours (Hull 1970) and we were interested in testing acute lethality only. Following treatment, all samples were acclimated to room temperature (~24 °C) for approximately 1 hour.

Rhizome freezing data collection

To determine the effect of cold treatments on rhizome viability, after treatment application all samples were removed from the plastic culture tubes and half of the rhizome segments (5) of each population's replicates were planted at ~2 cm depth in potting mix in plastic transplant trays (Vacuum Plug Tray, The H.C. Companies, Twinsburg OH). Trays consisted of ninety-eight 32 cm^3 cells for the individual rhizome segment. Propagation trays were maintained in light and uncovered at room temperature (~24 °C) in the laboratory and were watered to maintain even moisture (every ~3 days). We recorded binary success/failure to emerge, as well as days from treatment to emergence for each rhizome fragment sample.

The other half (5) of the replicates were submerged in 10 mL of deionized water in individual glass test tubes at ~24 °C for electrolyte leakage assessment. Electrolyte leakage, in which K ions play a critical role (Demidchik et al. 2014), is an accepted proxy for cell death and tissue damage and has been used to estimate frost injury in other rhizomatous plant species (Peixoto and Sage 2016). After 24 hours, each of the samples was removed from the water, and we recorded specific conductance (µS/cm) of each sample's water, using a benchtop conductivity meter (AI502 EC700, Apera Instruments LLC, Columbus OH). Each rhizome sample was then microwaved for

150 seconds to trigger maximal electrolyte leakage. Microwaved samples were then returned to the same water containers where they had been for the preceding 24 hours. After another 24 hours, samples were removed again and specific conductance measured. We calculated relative conductivity (RC), which represents the proportion of total possible electrolyte leakage that was induced by cold treatment application, using the equation:

$$RC = EC_t / EC_d$$

where EC = electrical conductivity (i.e., specific conductance), t = post-treatment, d = dead (microwaved). This, in turn, served as a proxy for the proportion of rhizome tissue damaged by the treatment.

Rhizome freezing statistical analyses

We established a relationship between rhizome emergence and RC across all populations and temperatures with a logistic model and extracted the lethal dose (LD90) value – the RC value at which at least 90% of rhizomes do not emerge. We then fitted a Gompertz curve to the RC responses of each population to the treatment temperatures. Each of these curves was then used to inversely predict the temperature at which the LD90 RC value was achieved, yielding each population's 90% lethal temperature (LT90) value. We then conducted stepwise linear regression of population LT90s on mean sample mass, as well as ecotype identity, MinT, MAP, and second order interactions, optimizing for the corrected Akaike Information Criterion (AICc) via backward selection. Mean sample mass was not subject to model reduction. The logistic model and LD90 extraction were performed in R (3.5.0; R Core Team 2018) using packages 'aod' (Lesnoff and Lancelot 2012), 'ggplot2' (Wickham 2016), 'MASS' (Venables and Ripley 2002), and 'popbio' (Stubben and Milligan 2007). Inverse prediction of LT90 and linear regression were performed with JMP Pro, Version 15 (SAS Institute, Inc., Cary, NC).

Seed freezing experiment

To conduct the seed freezing experiment, we selected the same 18 populations (9 agricultural, 9 non-agricultural) from the rhizome freezing experiment (Table 1), subsequently excluding 3 of them due to poor seed quality. Roughly 600 seeds per population were treated with commercial strength (7.4%) sodium hypochlorite (Clorox Regular-Bleach, The Clorox Company, Oakland CA) for 4 hours followed by a 1 hour water rinse (Atwater et al. 2016) to break dormancy. The prepared seeds were then sealed in the same type of plastic culture tubes as the rhizome segments and each set of samples (one capsule of ~600 seeds per population) was sealed in a plastic bag. We conducted several preliminary cold treatments using the same temperatures from the rhizome experiment. However, we observed no effect of these temperatures on germination percentage(GP) or mean time to germination (MGT) for any population.

Thus, seed freezing treatments were applied at two levels (-20 °C and -85 °C, at ~100% relative humidity) for 24 hours. This duration was chosen based on a preliminary trial yielding non-zero germination of a single population chosen at random. We chose these treatment temperatures as we found no change in germinability between 0 °C and -20 °C, and we were limited by equipment availability, as well as cooling capacities of the baths used in the rhizome experiment described above. For reference, the coldest air temperature ever recorded on Earth's surface is -89.2 °C at the Vostok Station, Antarctica (Turner et al. 2009). Thus, our treatment temperatures should be viewed more as proof of concept for seed cold tolerance and less as a simulation of Johnsongrass seed response to freezing in the field. In other words, if seeds are capable of surviving 24 hours at -85 °C, then range expansion is likely not limited by seed acute cold tolerance. The control was ambient temperature (24 °C) maintained for 24 hours. Following treatment, seeds from each population were distributed at random into 20-seed replicates and placed in Petri dishes (one dish per replicate) with water-saturated filter paper (Whatman 1003-055, GE Healthcare, Chicago IL). Randomly sampled distribution of treated seeds into replicates was done in order to account for differences in micro-environments that may have been experienced at different locations in the capsule. The 270 Petri dishes (18 populations × 3 treatments × 5 replicates) were arranged in a randomized complete block design in the laboratory. Each replicate dish of 20 Johnsongrass seeds was sealed with Parafilm M laboratory film (Sigma-Aldrich, St. Louis, MO) to prevent excessive moisture loss.

Seed freezing data collection

We recorded the proportion of seeds in each replicate that successfully germinated (GP), as well as the number of days elapsed between treatment and germination. We also derived a mean time to germination (MGT) in days per seed for each 20-seed Petri dish. Data were collected until no new germination occurred, which was within 12 days of treatment application.

Seed freezing statistical analyses

We conducted mixed effects linear regression models of GP and MGT on experimental and populations origin variables. The model included fixed effects of ecotype, treatment temperature, MinT, and MAP, block as a random effect, as well as all possible second order interactions among the fixed effects. We then performed backward model selection, removing non-significant predictors in order to optimize AICc. Block was the only factor intentionally conserved in both models. All statistical analyses on seed freezing data were performed using JMP Pro, Version 15 (SAS Institute, Inc., Cary, NC).

Data resources

Experimental data are provided in an associated file (Suppl. material 1: Table S1).

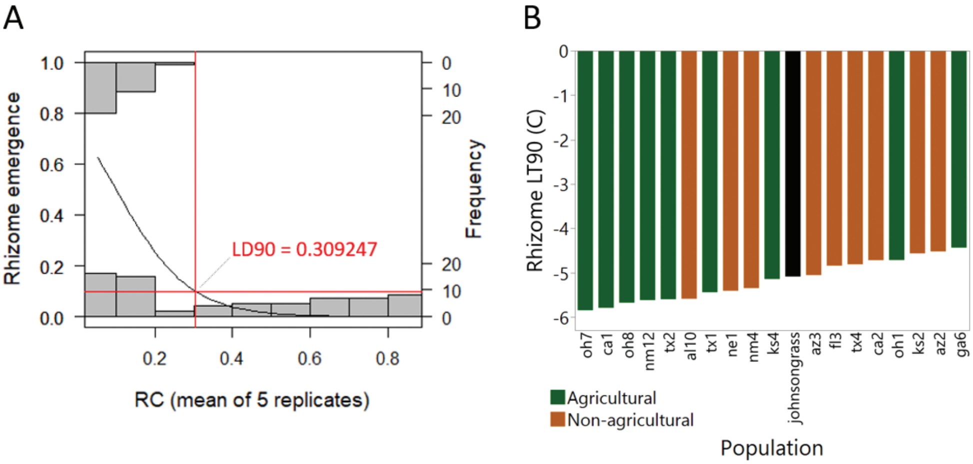

Figure 2. Plots of **A** the logit model of rhizome emergence response to 5-replicate relative conductivity (RC) means (p = 0.0081), yielding a lethal dose for 90% of samples (LD90) of 0.309247 and **B** population and whole species lethal temperature for 90% of samples (LT90) values in degrees C based on rhizome RC values, in ascending order.

Results

Rhizome freezing tolerance

No rhizomes emerged after the -10 °C treatment, but all other treatments (-6 °C, -4 °C, -2 °C) yielded partial emergence. Logistic regression of rhizome emergence on treatment temperature found a significant effect ($p < 0.0001$) with a predicted LT90 of -7.1 °C, which we calculated instead of the LT50 due to the baseline emergence rate (at the warmest temperatures) of ~50%. Furthermore, rhizome emergence was erratic within populations due to node viability or other uncontrollable qualities, making results too variable for direct estimates of population-level LT90. Therefore, we used the logistic relationship between RC and emergence (p = 0.0081, Fig. 2A) to establish a species-wide RC value as a proxy for 90% failure to emerge (LD90). This LD90 value for the effect of RC on emergence was 0.3093 for all Johnsongrass populations. We then used this proxy to derive an LT90 from each population's RC-temperature response curve. Unlike the emergence data, RC data for each population were less variable and correlated well with Gompertz sigmoidal curves ($R^2 = 0.94 \pm 0.01$).

Rhizome LT90 was generally lower among agricultural (-5.36 ± 0.17) than non-agricultural (-4.98 ± 0.13) populations, but this relationship was not significant (p = 0.079). Rhizome LT90 also did not correlate with MinT (p = 0.640), MAP (p = 0.848), or sample mass (p = 0.478). Population LT90 values ranged from -5.67 °C (OH-8) to -4.43 °C (GA-6), with overall Johnsongrass rhizome LT90 calculated at -5.08 °C (Fig. 2B).

Table 2. Effect tests of each linear model of seed germination percentage (GP) and mean germination time (MGT) as reduced via backward stepwise selection for optimized Akaike Information Criterion (AICc). Both response variables were log-transformed to meet model assumptions. Square brackets around variable names indicate variable locking prior to stepwise selection. Alpha level of significance indicated by *** = 0.0005, ** = 0.005, * = 0.05.

	$\log_{10}$(GP)				$\log_{10}$(MGT)			
	DF	SS	F	p	DF	SS	F	p
[Block]	4	2.041	3.809	0.0526	4	0.191	3.101	0.0800
Ecotype	1	0.940	1.753	0.1872	1	0.073	1.190	0.2768
MinT	1	2.541	4.741	0.0308*	1	0.170	2.760	0.0985
MAP	1	5.667	10.576	0.0014**	1	0.086	13.907	0.0003**
Treatment Temp	1	17.980	33.552	<0.0001***	1	2.187	35.590	<0.0001***
Ecotype*MinT	1	2.845	5.309	0.0224*	1	0.687	11.176	0.001**
MinT*MAP	1	14.554	27.159	<0.0001***	1	1.423	23.156	<0.0001***

Seed freezing tolerance

Colder treatment temperatures decreased both GP and MGT ($p < 0.0001$; Table 2). Across all populations, on average, 31.2% of seeds in the control group germinated at 22 °C. Seeds in the control group took an average of 0.45 days to germinate. The -20 °C treatment resulted in 27.7% germination overall. Seeds treated at -20 °C took an average of 0.88 days to germinate. The -85 °C treatment yielded 12.3% germination and took an average of 2.9 days to germinate.

There were marked population differences in germination percentage (GP) within and across treatments (Fig. 3). Across all temperatures, GP at the population level ranged from 4% (KS-4) to 59% (TX-2). The extremes in MGT across all temperatures were also represented by these two populations, ranging from 11.1 (KS-4) to 0.18 (TX-2). These two populations' composite GP and MGT ranks were not uniform across particular treatment temperatures. In the control group, AL-10 had the lowest mean germination at 1% while TX-2 was highest at 80%. At -20 °C, KS-4 had the lowest mean GP of 6%, while TX-4 was highest at 71%. At -85 °C, CA-2 had the lowest germination at 0% total, while OH-8 had the highest mean GP at 34%.

Effects of MinT, MAP, and ecotype on seed germination

We found an interactive effect of MinT and MAP on both GP and MGT ($p < 0.0001$ for both; see Table 2). Cold/dry and warm/wet climates were associated with greater and faster germination than cold/wet and warm/dry climates (Fig. 4). Agricultural and non-agricultural populations responded inversely to each other to home temperature in both GP ($p = 0.0224$) and MGT ($p = 0.001$) (Table 2). GP of agricultural populations increased by 1.4% per degree C of home MinT, while that of non-agricultural populations decreased by 2.5% per degree C of home MinT (Fig. 5A). Similarly, the

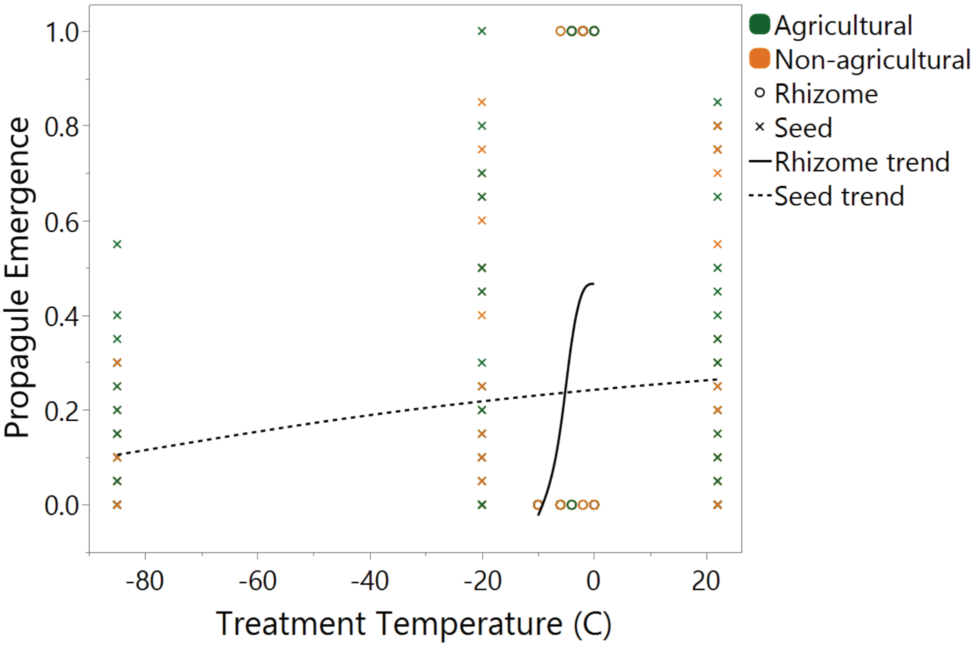

Figure 3. Seed and Rhizome emergence in response to their respective suites of treatment temperatures. Propagule type is indicated by marker shape and ecotype identity (agricultural vs. non-agricultural) is indicated by marker color. Markers allowed to overlap (not jittered) for legibility.

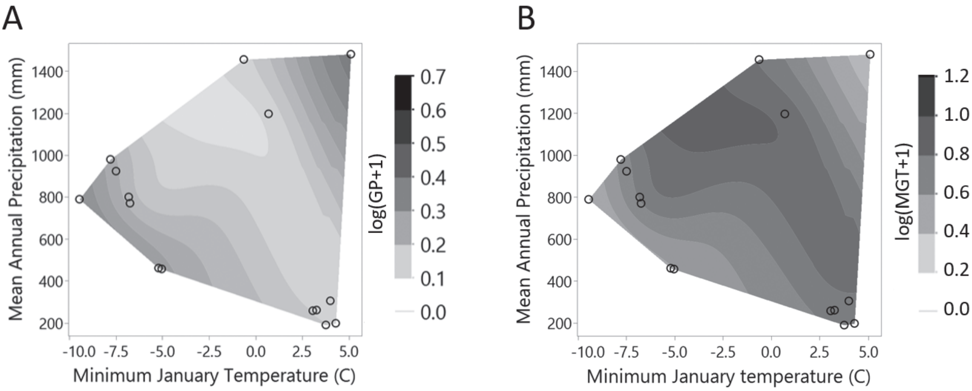

Figure 4. Interactive effects of **A** minimum January temperature (MinT) and mean annual precipitation (MAP) on germination percentage (GP) ($p < 0.0001$) and **B** MinT and MAP on mean germination time (MGT) ($p < 0.0001$) show greater and faster germination associated with populations from cold-and-dry and hot-and-humid climates. Both response variables are $\log_{10}$ transformed to meet model assumptions.

MGT of agricultural populations increased by 0.53 days for every degree of home MinT, while that of non-agricultural populations decreased by 5 days for every degree of home MinT (Fig. 5B).

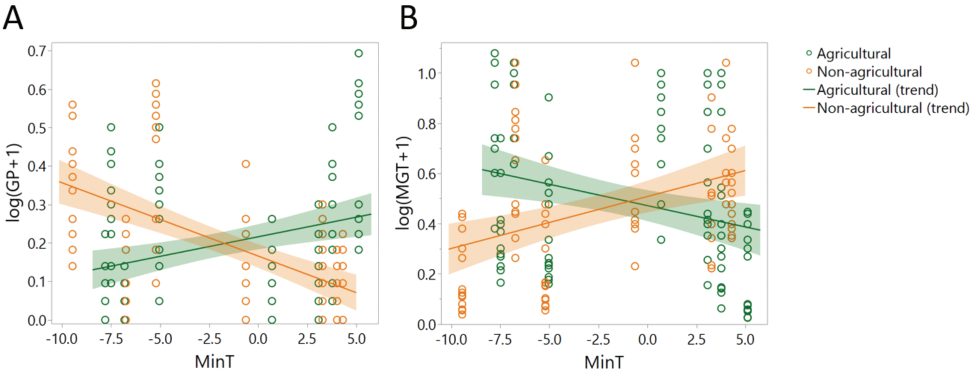

Figure 5. Interactive effects of **A** ecotype identity and minimum January temperature (MinT) on seed germination percentage (GP) ($p = 0.0224$) and **B** ecotype identity and MinT on mean germination time (MGT) ($p < 0.0001$). Both response variables are $\log_{10}$ transformed to meet model assumptions.

Discussion

We found that both Johnsongrass seed and rhizome are affected by exposure to acute treatment temperature minima, but on very different scales. While rhizome emergence showed a sharp decline from ~50% emergence to non-viability in the vicinity of -5 °C, seed germinability declined very gradually from ~25% to 10% across the gradient of 22 to -85 °C. The rhizome survival threshold of approximately -5 °C confirmed Hull's (1970) finding of sharp decreases in rhizome survival between -3 °C and -5 °C to the point of no viability, and appears consistent across the broad geographic range. Thus, we saw that overwintering potentials of seed and rhizome are vastly different, implying seed survivability in climates north of Johnsongrass's current range, far beyond known non-perenniating populations in southern Ontario (Warwick and Black 1983). Additionally, population differences based on home climate and ecotype were observed in seed, but not in rhizome. While these differences may be adaptive, it is all but certain that successful germination following -85 °C freezing treatment is not a trait selected for in the landscape, as Johnsongrass does not experience such extremes anywhere in, or near, its range; nor are there many places on Earth with such low temperatures. Interestingly, the impacts of home MinT on seed GP and MGT were mediated by ecotype identity, which may shed light on agricultural practices selecting for traits related to temperature and, by association, day length. The twofold (50% vs. 25%) difference in baseline (control) emergence of rhizome over seed, in conjunction with the latter's vast numerical superiority, echoes Atwater et al. (2017) conclusion that rhizome is more efficient than seed on a per-propagule basis, while seed is more efficient on a per-unit-carbon basis.

We found no differences in seed germination or rhizome emergence response – and therefore no differences in cold tolerance – to freezing treatments based on home MAP and MinT. There were, however, inherent differences in seed germination response based on home MAP and MinT. This yielded a response surface where cool/

dry and warm/humid origin Johnsongrass populations germinated more and faster than cool/humid and warm/dry origin populations. It is possible that reduced and delayed germination on dry sites may be a conservative strategy selected for by drought stress, which can be especially damaging for seedlings, compared to seeds or mature plants (Schwinning et al. 2017). Meanwhile, an adaptation for proportionally higher germination in cold habitats may buffer against inevitable partial die-off in late frosts. Though associated more with growing season length than late frosts specifically, such a strategic adaptation has been found in the introduced range of the invader *Ambrosia artemisiifolia* (Leiblein-Wild et al. 2014). Earlier germination and leaf-out has been known to correspond to quicker recovery following freezing damage (Menzel et al. 2015). Therefore, one explanation for no difference in MGT based on home MinT could be that seedling Johnsongrass is unable to resprout following frost damage. This vulnerability may also contribute to inefficiencies in adapting to conditions at its northern range limits (Fletcher et al. 2020). Finally, earlier leaf-out as part of overall extended leaf phenology is a common strategy among invasive plants (Fridley 2012), though its ultimate utility in carbon gain is diminished at increasingly northern latitudes (O'Connell and Savage 2020).

No differences were found between agricultural and non-agricultural populations' seed or rhizome responses to freezing treatment temperatures, indicating no differences in cold tolerance based on ecotype identity. However, we again found inherent differences in GP and MGT based on home MinT as mediated by ecotype identity. Non-agricultural populations germinated more and faster than agricultural ones when originating from colder climates; however this did not translate to any differential response to our cold treatments. Given the smooth decrease in germinability from +22 °C to -85 °C treatments across all populations, it makes sense that population differences based on a MinT range of -10 °C to +5 °C are unrelated to survival of -85 °C or even -20 °C treatments. Tolerance of the extreme cold could not have been selected for in the landscape, as Johnsongrass seed does not encounter these temperatures. Unfortunately, we were limited by available equipment to test temperatures between -10 and -85 °C. We had posited that any differences in cold tolerance between ecotypes could be driven by energy assimilation and storage from a more favorable preceding season; however, this could not have been the case for seed, as we accounted for maternal effects by using only germplasm that had been grown out in a common environment for a generation.

Cold tolerance differences between seed and rhizome were so vast that they cannot be compared by LT90 values. Seed GP approached 0.1 (analogous to LT90) around -85 °C and no colder treatment was available, meaning that a true dose response curve could not be built for seed as it was for rhizome. This extreme cold tolerance across Johnsongrass populations informed us that seed freezing is likely not range limiting. Given no origin MAP or MinT differences in rhizome LT90, we also cannot test whether the annual climate niche is significantly different between populations. Instead, our evidence points to propagule pressure and phenology as likely factors of northern range limitation. Given the relatively high winter temperatures that rhizomes cannot survive, rhizome segments likely cease to be feasible propagules for range expansion in regions with climates similar to southern Ontario, where populations persist only via seed (Warwick and Black 1983). The general uniformity of rhizome LT90s across the species range suggests that rhizome cold tolerance is unlikely to adapt to colder climates in the future. Because we used only one cold acclimation regime across treatments, it is also possible that differences in acclimation capacity are present between populations, such as found in *Miscanthus* by Peixoto and Sage (2016). However, we have less reason to suspect such an effect in Johnsongrass, as *Miscanthus* is known to have a wider overall range in cold tolerance among genotypes (Fonteyne et al. 2016). It should also be noted that, even though GP values were low overall, the stochastic nature of these reductions and the very high significance level of model effects across 18 populations tell us that these correlations are robust. It is not unexpected to observe low overall seed germinability in weedy and invasive species that have evolved seed dormancy.

Dormant and non-dormant seeds are clearly not range limiting to Johnsongrass as a species, nor limited based on ecotype or home climate. In other words, seed from anywhere in the North American range can survive winter temperature minima anywhere on the continent. Given that Johnsongrass persists in places with colder winter temperatures than the rhizome LT90 of -5 °C, thermal dynamics of soil are clearly a factor that prevents us from simply predicting cold temperature range limitation. Lack of apparent climate or ecotype adaptation of rhizome cold tolerance tells us that this may be a stable trait within the species, while an expanding "annual range" beyond the perennial range is feasible. However, even though seeds may always be cold tolerant, seedlings are likely to be much more vulnerable to stressors such as late frosts and droughts (Olson et al. 2018). Conceivably, more and faster germination as an adaptation to colder climates could be based on limited photosynthetically active radiation (PAR) of shorter summers. But why is this only seen in non-agricultural populations, while the inverse is true of agricultural ones? One driver of colder origin agricultural seed germinating less and slower than its non-agricultural counterpart could be greater winter/spring cold exposure in cropland due to bare soil (Snyder et al. 2015). It is also possible that early application of post-emergence herbicides could select for later germination. For example, a genetic link between herbicide resistance, dormancy and germination behavior is seen in the weedy grass *Alopecurus myosuroides* (Délye et al. 2013). Other agricultural factors could be at work, as seed phenology is known to be tremendously adaptive to cropping systems (Batlla et al. 2020). Molecular regulation of cold tolerance is currently an ongoing investigation with much progress made in understanding both the stress signaling and acclimation response involved (Ding et al. 2019).

One of the primary challenges in interpreting rhizome cold tolerance and forming hypotheses about continental distributions is the interaction of climate change with snow cover and, thereby, insulation of soils in winter. Rhizome carbohydrate storage, bud formation, survival, and phenology of spring emergence are known to be sensitive to winter snow depths (Lubbe et al. 2021). Specifically, reduced snowpack as a result of winter warming has been seen as particularly injurious to rhizomatous species populations, suppressing their competitive ability in herbaceous communities (Lubbe and Henry 2021). Winter warming, rather than summer warming, has been linked to major shifts in grassland primary production, species composition, and soil respiration (Kreyling et al. 2019), suggesting that future studies should also focus on temperature minima at population origins to study community assembly, as well as improved measures of perenniation, budbank, and multi-trait assessment (Lubbe et al. 2021).

Conclusion

By uncovering drastic differences in cold tolerance and between organs and populations, we are able to better understand their potential contributions to species distributions. We can begin to deduce which organs may or may not be limiting to overall plant stress tolerance and whether there are other physiological or phenological drivers of known range limits. Likewise, we can narrow possible drivers of range limitation and connect them to spatially explicit habitat parameters. However, we must be careful not to conflate experimentally isolated stress limits with distribution boundaries (Curtis and Bradley 2016). In this example of Johnsongrass seed versus rhizome cold tolerance, it becomes clearer whether or not a trait could have been acquired through selection. Namely, we see that, while seed is virtually unlimited by temperature minima in North America, we should not assume a lack of northern range limit. Likewise, we found rhizome cold tolerance to be less than what the perennial range would suggest, congruent with Curtis and Bradley's (2016) findings across many species. Furthermore, this approach has provided us with leads in terms of which organs, life stages, and stressors to investigate further. It also allowed us to characterize seed germinability and germination performance of different Johnsongrass ecotypes over a broad range of home climates.

We see that not all propagules of a plant respond similarly to all stresses – cold temperatures being a key example. In studying and managing invasive plants, this can inform our understanding of likely dispersal vectors. Our findings on Johnsongrass, in corroboration with Atwater et al. (2017), tell us that seed is a far more efficient, stress tolerant, and easily transported propagule than rhizome. This implies that, if most Johnsongrass populations are established by seed, we should expect ample genetic variation within and among landscapes compared to stands where clonal propagation is likely to occur. This genetic diversity contributes to the known intraspecific variation across the continent (Atwater et al. 2016, 2017; Sezen et al. 2016; Fletcher et al. 2020; Kelly et al. 2020), as well as its likelihood of adaptation to new habitats. At the same time, our study suggests some hard limits to cold adaptation, which is both ecologically interesting and informative for invasion risk assessment.

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Supplementary material 1

Table S1

Authors: Vasiliy T. Lakoba, Gregory E. Welbaum, John R. Seiler, Jacob N. Barney

Data type: propagule emergence

Explanation note: A table of emergence response by population, temperature, and propagule type.

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RESEARCH ARTICLE

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Spatial dynamics of spotted lanternfly, *Lycorma delicatula*, invasion of the Northeastern United States

Rachel T. Cook[1], Samuel F. Ward[2], Andrew M. Liebhold[3,4], Songlin Fei[1]

1 *Purdue University Department of Forestry and Natural Resources, 715 West State Street, West Lafayette, IN, USA* **2** *Mississippi State University Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, 100 Old Highway 12, Clay Lyle Entomology Building, Mississippi State, MS, USA* **3** *USDA Forest Service Northern Research Station, 180 Canfield Street, Morgantown, WV, USA* **4** *Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, 165 00, Praha 6 – Suchdol, Czech Republic*

Corresponding author: Songlin Fei (sfei@purdue.edu)

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Abstract

Spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is a non-native planthopper that recently established in the Northeastern United States. Little is known about the spatial dynamics of its invasion and key drivers associated with its regional spread. Here, using field survey data from a total of 241,366 survey locations from 2014–2019 in the eastern USA, we quantified rates of SLF spread and modeled factors associated with the risk of SLF invasion. During the study period, SLF invasion appears to be associated with both short- and long-distance dispersal. On average, the number of newly invaded counties per year increased since initial discovery, with 0–14 long-distance dispersal events per year and median jump distances ranging from 55 to 92 km/year throughout the study period. Radial rates of spread, based on two of the three analysis methods applied, varied from 38.6 to 46.2 km/year. A Cox proportional hazards model suggested that risk of SLF invasion increased with a proxy for human-aided dispersal, human population per county. We anticipate that SLF will continue to spread via both long- and short-distance dispersals, especially via human activities. Efforts to manage SLF populations potentially could target human-mediated movement of SLF to reduce rates of spread.

Keywords

Biological invasion, Cox proportional hazards, spatiotemporal, invasive species, radial spread

Introduction

Though most non-native pests fail to establish after arrival, those that successfully found reproducing populations can subsequently spread via a coupling of population growth with dispersal. The dispersal of many invading species is characterized by the simultaneous occurrence of local diffusion and occasional long-distance dispersal (Hastings 2005; Liebhold and Tobin 2008). Information on what factors drive spread of a non-native pest can guide management to contain its populations and reduce their impacts to ecosystems and economic costs (Sharov and Liebhold 1998; Liebhold and Kean 2019). Understanding the factors that drive spread is particularly important for newly established species, for which dispersal behaviors and population growth characteristics are often unknown.

Spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is a non-native planthopper recently established in the United States. The species is native to southeast Asia, but recently invaded the USA in Berks County, Pennsylvania in 2014 (Barringer et al. 2015). Spotted lanternfly (SLF) is univoltine and lays egg masses on a variety of surfaces, including tree bark, stone, motor vehicles, and trains (Urban 2019). In addition to indiscriminate egg deposition, SLF also has a wide breadth of host use. This pest feeds on over 70 species of herbaceous and woody plants belonging to over 20 families, though it prefers tree of heaven (*Ailanthus altissima*), especially as a late instar (Dara et al. 2015; Parra et al. 2017). Notably, SLF feeds on apple (*Malus* spp.) and grape (*Vitis* spp.), both important agricultural plants in the Northeastern USA. Feeding on grape has reportedly resulted in lower fruit quality, less fruit production, and elevated mortality, though minimal impacts to fruit tree health have been reported (Urban 2019). The most conspicuous impact of SLF in forests is the accumulation of honeydew in the understory, which results in sooty mold growth that limits photosynthesis and growth of understory plants (Ding et al. 2006; Parra et al. 2017). There is also evidence that aggregation of SLF can cause weeping wounds on trees, resulting in crown dieback (Dara et al. 2015). While detrimental impacts on tree of heaven might be beneficial due to its status as an invasive plant, SLF is considered a serious pest due to its negative impacts on agricultural crops and native trees.

Despite regulations by the state of Pennsylvania that prohibit movement of any SLF living stage (e.g. egg masses, nymphs, adults) or material potentially harboring the pest (e.g. firewood, nursery stock, etc.) outside of a quarantine area, SLF has spread from Pennsylvania to seven surrounding states as of 2019 (Fig. 1). Because SLF was detected in the USA recently, there is little information on how this species spreads or what drives its invasion. Though the body of knowledge on this insect is growing, many aspects of SLF spread, especially the role of environmental drivers, are unknown. Elucidating how this pest spreads can inform future management and survey efforts.

The ranges of introduced species are influenced by a multitude of anthropogenic factors and habitat features. For SLF, climatic niche models indicate that half of the USA, including most of the New England, Mid-Atlantic, and Pacific Coast states, is at risk of invasion (Wakie et al. 2020). While these climatic niche models provide valu-

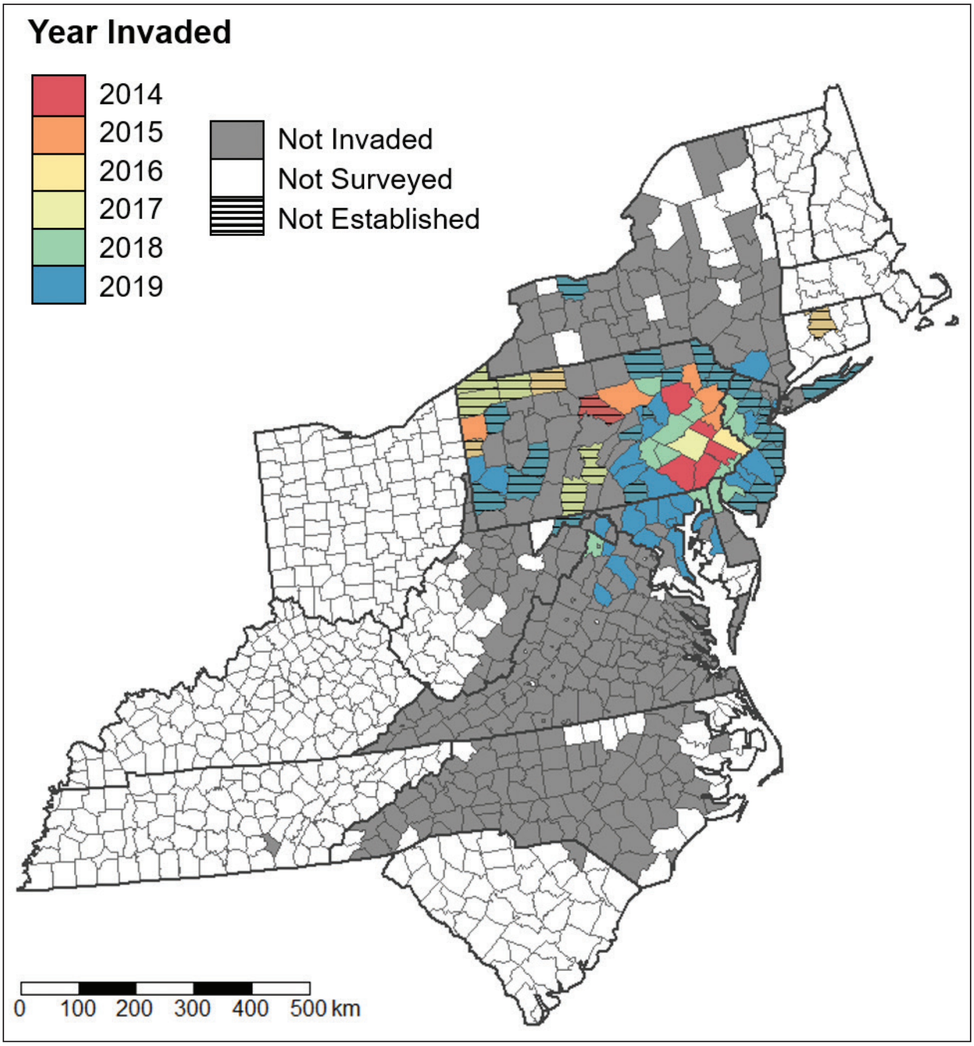

Figure 1. County-level distributions of spotted lanternfly (SLF) in the eastern USA. Distribution of SLF detections and establishments by year based on USDA Animal and Plant Health Inspection Service and Pennsylvania Department of Agriculture visual survey data. Counties with hash marks had SLF detections that failed to establish. We define a county as invaded when the county experiences at least two consecutive years of SLF detection, and define year invaded as the first of those two consecutive years. Counties with white color were not surveyed.

able information on where SLF can potentially establish, analyses of spread can provide insight into how quickly SLF will arrive and what habitat and/or anthropogenic factors affect the dynamics of SLF spread. SLF has undergone several long-distance dispersal events that likely resulted from human-mediated transportation (Eddy 2018; Scheid 2020). Tree of heaven is also more abundant in urban areas, and thus human activities may increase both propagule pressure and habitat suitability. However, the rate of spread, including the frequency and distance of long-distance dispersal events, and drivers of spread have not been quantified. Therefore, we investigated how anthropogenic and habitat factors are related to SLF spread.

We analyzed the known geographical distribution of SLF (2014–2020) in the USA to quantify its rates of spread and identify factors that influence its invasion risk. Our goals were to: 1) describe the patterns of SLF spread following the initial detection in 2014, and 2) identify key drivers that are associated with SLF spread. For our second goal, we used known occurrences of SLF in conjunction with habitat and anthropogenic variables to determine the most important factors driving county-level invasion risk across the study area, defined below. We hypothesized that anthropogenic factors are important drivers of SLF spread, given the ability of this insect to lay inconspicuous eggs on a variety of materials, including motor vehicles and trains (Urban 2019).

Methods

The SLF distribution data analyzed in this study were derived from visual surveys conducted from 2014–2019 by the US Department of Agriculture, Animal and Plant Health Inspection Service (APHIS) and the Pennsylvania Department of Agriculture (PDA). We also used SLF county-level presence data for 2020 from the New York State Integrated Pest Management Program (Cornell 2021). Survey data include geospatial coordinates for survey locations as well as the number of SLF observed (if any). A total of 241,366 survey locations were obtained for this study (Suppl. material 1). Given the irregularity of survey locations and potential biases (e.g. surveys at expected SLF locations) and to render data at an equivalent scale as the 2020 presence data, we converted counts to county-level presence/absence records and used county as our unit of analysis.

The survey data contained many points that we identified as failed establishments in which SLF were observed in a county in a given year but were absent in surveys of the same county in subsequent years. These detections were likely either populations that failed to establish or regulatory incidents, such as dead SLF adults found in transported materials, and thus we did not treat them as invasions. Hereafter, we refer to detections as establishments plus failed establishments and establishments as only populations that persisted for more than one survey year in consecutive years within a county. Moreover, we categorized each invaded county in year *n* as contiguous or non-noncontiguous based on the presence or absence, respectively, of an invaded neighboring county in year *n*-1.

Described below are methods we used to 1) determine aspects of spread dynamics, such as jump distances and spread events into contiguous vs. non-contiguous counties, 2) compare three methods of estimating spread rates, and 3) fit a Cox proportional hazards model estimating time-to-invasion as a function of variables representing spatial proximity to existing SLF populations (henceforth referred to as spatial proximity), habitat suitability, and anthropogenic influences. Our study area was defined as the area of the eastern USA invaded in 2019 plus a buffer distance of 355 km, equal to the maximum observed jump distance (see "Characterization of spread events" in

Methods). This study area was used for all subsequent analyses. Counties, which are the level at which quarantines and other management decisions are set, served as the unit of analysis for all analyses. All analyses were conducted using R version 4.0.2 (R Core Team 2020).

Characterization of spread events

To characterize spread, we quantified the number of yearly spread events into contiguous and non-contiguous counties, as well as the distribution of jump distances. Jump distance is defined as the distance between establishments or detections in non-contiguous counties in year *n* and the nearest previously invaded county in year *n*-1. We estimated jump distances for every newly invaded county by calculating the distance to the closest previously invaded county, as assuming new SLF establishments originate from the closest previously invaded county provides a conservative estimate. Distances were measured using county centroids. We repeated this process for each year, and summarized the distribution of jump distances (e.g. median, minimum, maximum). To determine if spatial proximity is related to whether or not a detection became an establishment (i.e. an invasion persisted), we separated jump distances by establishments and failed establishments and used a Mann-Whitney U test to compare the distribution of jump distances between these two groups.

Spread rates

Because little is currently known about SLF spread patterns and different approaches can provide variable estimates of annual spread (Tobin et al. 2006), we compared three methods to calculate spread rate described by Gilbert and Liebhold (2010). The purpose of our comparison of these methods is to provide a range of possible spread rates as well as to determine robustness of each when applied to an insect at early stages of invasion.

The first method is to apply regression of the distance (centroid to centroid Euclidean distance) of every county with positive establishment from the point of initial detection (Berks County, PA) as a function of years since initial detection (2014). The resulting slope of the estimated regression equation estimates the radial rate of spread measured in distance/year. The second method is to regress the square root of the invaded area (estimated by summing the area of invaded counties in each year) divided by π on time. The resulting slope of the estimated regression line estimates the radial spread rate in distance/year (e.g. effective range radius; Shigesada et al. 1995). Last, we calculated the average distance between invasion boundaries in consecutive years along radii emanating every 0.5 degrees from the centroid of Berks County, PA. We used radii at a frequent degree interval to obtain a high-resolution estimate of yearly distance between boundaries. We found invasion boundaries by fitting a convex hull polygon to the area of invasion in each year and subsequently converting the polygon edges to lines. The convex hull polygon in each year was stretched to the edges of non-contiguous invaded counties. The resulting average distance between boundaries on each radius between consecutive years can be used to estimate the annual radial spread rate (e.g. boundary displacement rate). Due to the nature of fitting a convex hull polygon around invaded counties, we used county boundaries as opposed to county centroids to calculate distances in boundary displacement estimations. In summary, distance regression is based on distance and year of sampling points from the origin where the species was first detected, while effective range radius considers area invaded over time. Boundary displacement estimates distance between invasion boundaries in consecutive years.

Dispersal kernel estimation

Dispersal kernels estimating risk of invasion as a function of distance have been developed for other invading forest insects (Orlova-Bienkowskaja and Bienkowski 2018). Given interspecific variation in spread rates (Fahrner and Aukema 2018), however, we estimated a SLF specific dispersal kernel, which, in turn, should enable more reliable estimates of the effects of SLF spatial proximity on invasion risk. Our analysis used 2015–2019 county-level SLF survey data from USDA APHIS and PDA and follows methods from Kovacs et al. (2010). A negative exponential function was used to model the probability, p, of each non-invaded county in the study area becoming invaded on an annual basis from 2015–2019:

$$p_{i,j} = e^{-ad} \quad (1)$$

where a is the parameter we sought to estimate and d is the distance in kilometers to a previously invaded county. To estimate a, we simulated county-level spread starting from the five initially invaded counties in 2014 using values of a between 0.01 and 0.10 in 0.001 intervals.

To simulate spread for a given a value, we calculated the centroid to centroid Euclidean distance from each non-invaded county i in year n to each invaded county j as of year n-1, as each county j invaded as of year n-1 could serve as a source for invasion into county i in year n. The distances from county i to each invaded county j were input into Equation 1, producing an estimate, p, for the probability of SLF invading from each county j. This probability value was then used to parameterize a Bernoulli distribution such that the probability of an event was equal to p. We then took a random draw from that Bernoulli distribution in which a draw of 1 or 0 would indicate invasion or non-invasion, respectively. This meant that there were x draws for each non-invaded county i, where x = number of invaded counties in year n-1. If any draw produced a 1, the county was categorized as invaded for the rest of the simulation (i.e. counties could not become uninvaded).

A single iteration of this process produced a simulated, county-level invasion at annual time steps (2015–2019) that may or may not have reflected the realized invasion. For each a value, we conducted 500 iterative simulations, starting with the initially invaded counties in 2014 and forecasting spread to 2019. Results were summarized with accuracy values - false negatives and positives, and true negatives and positives - compared with the actual invasion data from 2015–2019. We selected the value of a that simultaneously resulted in the lowest number of false negatives and false positives when comparing actual spread to predicted spread.

Invasion drivers

Cox proportional hazards models can be used to estimate survival time based on predictor variables, including both static and time-varying predictors (Thomas and Reyes 2014). If we equate survival to a county persisting without invasion, we can use Cox proportional hazards models to evaluate which factors explain variation in time-to-invasion. Therefore, we used a Cox proportional hazards time-to-invasion model to evaluate potential drivers of SLF invasion at the county level, in a manner analogous to the implementation by Jules et al. (2002) and Ward et al. (2020).

The Cox proportional hazards model quantifies the probability of invasion at each one-year time step. Time steps ranged from 2014–2015 to 2018–2019. Predictor variables included static habitat variables (Suppl. material 4) and one time-varying predictor, spatial proximity. To quantify spatial proximity, we first used Equation 1, setting $a = 0.045$ (i.e. determined from the dispersal kernel estimation process described above; see "Invasion drivers" in Results) and d as the distance in kilometers between each uninvaded county to all previously invaded counties. Spatial proximity, denoted by *SpatialProx*, was then calculated for each county:

$SpatialProx_i = 1 - \prod(1 - p_{i,j})$. (2)

The other predictors included two anthropogenic variables and six habitat variables. The anthropogenic variables were human population from the U.S. Census and road density calculated by Liebhold et al. 2013 from the ArcGIS World Transportation reference layer (Suppl. material 2) and each was considered a proxy for human-aided dispersal. The six habitat variables included forested area and five host availability terms expressed as basal area, host trees per acre, number of host trees per county, tree of heaven occurrence, and canopy cover (Suppl. material 3). Forested land was obtained from the US Forest Service FIA MapMaker online data query system (https://www.nrs.fs.fed.us/fia/data-tools/mapping-tools). Percent forest canopy cover was obtained from the Forest Service's cartographic tree canopy cover product (USDA Forest Service 2016).

Host basal area and numbers of host trees per acre and county were obtained from the Forest Service's Forest Inventory and Analysis (FIA) program, using a published list of known SLF hosts from Barringer and Ciafre (2020). The FIA program is a long-term forest inventory program with one 0.40 hectare sample every 2,428 hectares, with most counties partially assessed annually since 2000. FIA assesses forest areas defined as at least 37 meters wide and 0.40 hectares in size, covered by at least 10% trees (Bechtold and Patterson 2005). We obtained plot-level basal area and stem density per acre from FIA records from 2015–2017. To estimate these variables at the county-level, we aggregated each by the summed county plot area for every known host with available FIA data by species code, obtained from the National Core Field Guide (USDA Forest Service 2019). We then estimated the number of each host species in a county by multiplying the estimated number of trees per acre by the total acres of forested land in each county. Because FIA only surveys forested areas, and tree of heaven is often found in developed or urbanized areas, a number of tree of heaven observations were downloaded separately as point data from EDDMapS (EDDMapS 2021) and aggregated to the county level by summing up the number of observations per county.

Prior to model development, we quantified pairwise correlations between our predictors to check for collinearity (defined as Pearson's product moment correlation coefficient ≥ 0.70). Based on this step, we removed road density and number of host trees per county due to collinearity with human population and forested area, respectively. We removed these two variables as opposed to human population and forested area because in preliminary models, they were more strongly associated (i.e. occurred in models with lower Akaike Information Criterion values) with SLF time-to-invasion than their co-varying counterparts. We then refined the model by applying a backward selection procedure that iteratively removed the variable associated with the highest *p*-value and refitting the model until only statistically significant predictors remained.

Results

Characterization of spread events

There was overall an upward trend in the number of newly invaded counties every year since initial discovery, although some counties contained failed establishments. There was a drop in counties with establishments in 2016 and 2017, while the highest number of establishments was observed in 2019 (Table 1). Similar to the number of newly invaded counties per year, number of counties with failed establishments generally increased across the study period and peaked in 2019. The highest percent of counties with failed establishments occurred in 2017, with 83% of detections failing to establish. The median yearly jump length into counties with detection and establishment ranged from 46 to 73 km and 50 to 92 km, respectively.

We did not find a significant difference between distributions of jump distances in established populations vs. failed establishments. Median jump distances across all years in failed establishments and established populations were 55 km and 71 km, respectively. A Mann-Whitney U test showed the distributions in the two groups did not significantly differ (W = 706, $p = 0.46$).

Current data suggest that the SLF invasion began in eastern Pennsylvania, and many of the counties invaded in the surrounding area of eastern and central Pennsylvania were contiguous with previously invaded counties (Fig. 2). In contrast, several populations in western Pennsylvania and northern Virginia resulted from invasion into non-contiguous counties, indicating long-distance jumps. There were no newly invaded non-contiguous counties in 2016 or 2017. Trends in the number of both contiguous and non-contiguous counties tracked the overall number of counties invaded, starting out low and increasing in 2018 and again in 2019 (Table 1). However, there were overall fewer non-contiguous counties invaded than contiguous counties across all years.

Establishments showed similar patterns in numbers of new counties invaded and jump distances by year (Table 1), with lower values from 2014 to 2017 and an increase

Table 1. Spread events summary. Number of observed contiguous (having at least one previously invaded neighboring county at time of invasion) and non-contiguous (having no previously invaded neighboring counties at time of invasion) newly invaded counties per year and median jump distances between invaded and uninvaded counties between consecutive years for both the non-persistent and the persistent counties.

	2014	2015	2016	2017	2018	2019	Total
Counties with detections (n)	5	6	4	6	18	47	86
Counties with establishment (n)	5	5	1	1	15	27	54
Counties with failed establishments (n)	0	1	3	5	3	20	32
% of Counties with failed establishments	-	16.7	75.0	83.3	16.7	42.6	-
Median jump length (km) into counties with detection	-	137.4	100.5	79.6	104.5	46.5	-
Median jump length (km) into counties with establishment	-	54.5	49.9	69.5	91.7	57.8	-
Counties with contiguous invasion	5	3	1	1	12	13	35
Counties with non-contiguous invasion	-	2	0	0	3	14	19

in 2018 and 2019. Median jump distances were greatest in 2017–2018 in the established counties, and were greatest in 2014–2015 in counties with detections. The year with the highest number of counties with newly established populations was 2019, whereas the year with the largest median jump distance (92 km) was 2018. Median jump distances were generally higher into counties with detections than counties with establishments. The overall maximum jump distance was 355 km into Mercer County in northwest Pennsylvania (Fig. 3), while the median jump distance was 55 km for detections and 71 km for establishments.

Spread rates

Estimated spread rates varied from 15–46 km per year among our three methods. Spread rate estimated by effective range radius was 46.2 km/year (SE = 7.19 km, 95% CI 26.26-66.20; Fig. 4A). Spread rate was estimated at 15.2 km/year (SE = 6.40 km, 95% CI 2.35-28.03) using distance regression (Fig. 4B). Spread rate estimated by average boundary displacement (averaged over all years) was 38.6 km/year (range 0 to 75 km; Fig. 4C), which was approximately 10 km less than estimated by effective range radius. The median boundary displacement across all years was 20.8 km/year. There was no difference in invaded area boundaries between 2016 and 2017, because the only newly invaded county was within the existing invasion boundary.

Invasion drivers

The best fitting value of *a* in the exponential dispersal kernel (*Equation* 1) was 0.045, which simultaneously resulted in the lowest number of false negatives and false positives. We used this value to estimate spatial proximity in the Cox proportional hazards model.

In the final Cox proportional hazards model, the hazards ratios for both spatial proximity and human population were greater than 1, indicating a positive relationship with increased risk of invasion (Table 2). Spatial proximity was identified as the strongest predictor (i.e. highest *Z*-value) with a notably high hazards ratio of ~40, followed by human population. No other covariates were statistically significant.

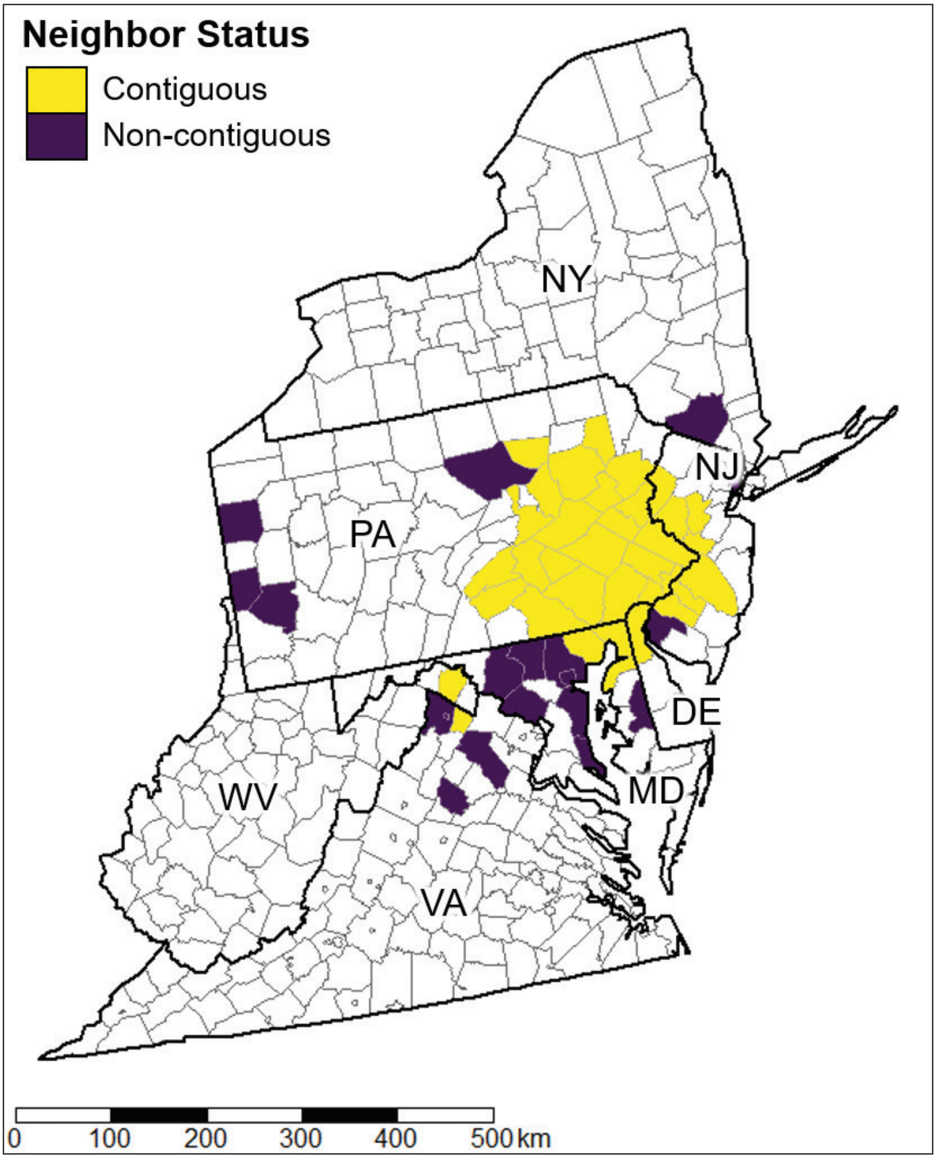

Figure 2. Contiguous and non-contiguous establishments of spotted lanternfly. Spatial distribution of contiguous (having at least one previously invaded neighboring county at time of invasion) and non-contiguous (having no previously invaded neighboring counties at time of invasion) counties across the study area.

Table 2. Final Cox proportional hazards model summary. Summary statistics from final Cox proportional hazards model predicting time-to-invasion of SLF at the county level in the study area.

Predictor	Estimate (coefficient)	SE	Z	*p*-value	Hazards ratio (95% CI)
SLF spatial proximity	3.70	0.286	12.94	<0.0001	40.29 (23.01-70.54)
Human population	0.28	0.126	2.22	0.0265	1.32 (1.03-1.69)

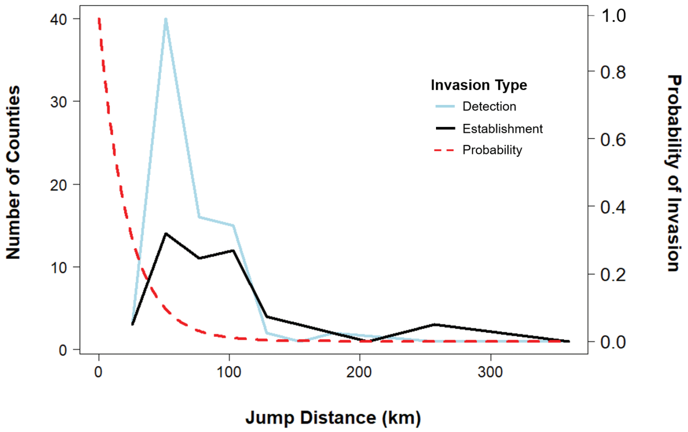

Figure 3. Jump distance distributions and probability of invasion by spotted lanternfly (SLF). Line graph of observed jump distances (the distance between new establishments in year *n* and the nearest previously invaded county in year *n*-1) for every newly invaded county for both establishments (black) and detections (blue). The red line indicates the probability of invasion by distance, based on the estimated SLF-specific negative exponential kernel function $p_{ij} = e^{-0.045d}$.

Discussion

Spread of invasive species is often characterized by both short- and long-distance dispersal. In many systems, short-distance dispersal is caused by the natural movement of organisms (e.g. flight behavior) while long-distance dispersal is caused by accidental human movement (Hastings et al. 2005). Even small amounts of long-distance dispersal can result in greatly elevated rates of spread (Shigesada et al. 1995). So far in the SLF invasion, movement appears to consist of both short- and long-distance dispersal. Little is known about natural dispersal in this species. Our results suggest, however, that risk of long-distance movement increases with human population density, likely reflecting the propensity of SLF to become associated with objects transported by humans, such as when SLFs oviposit onto train cars and motor vehicles (Urban 2019).

A higher number of new establishments occurred in contiguous than in non-contiguous counties, but several long-distance jumps were observed and the frequency of jumps appears to be increasing (Table 1). Human-mediated long-distance dispersal events are responsible for spread outside of the center of invasion, allowing for invasion of a larger geographic area than would be possible via insect movement alone. For example, the established population in northern Virginia (Frederick County) is believed to have originated from shipments from a stone yard in Pennsylvania (Eddy 2018). As SLF spreads, there may be increases in both long- and short-distance movement due to increases in numbers of source populations or increases in population size. Indeed, there have been additional long-distance dispersals beyond our study period (2014–2019), such as the discovery of SLF in Switzerland County, IN in July 2021 and the identification of SLF in a bug collection at the Kansas State Fair in early September

2021 (Edwards 2021; Indiana DNR 2021). A Mann Whitney U test showed no significant difference between jump distance distributions in detected but non-established vs. established populations, indicating that jump dispersal events are not necessarily more likely to persist if they are closer to the point of establishment (i.e. have closer spatial proximity). Ranges of jump distances were visually similar in range for both detected and established populations (Fig. 3), signifying that established jumps went at least as far as jumps that failed to establish. Shigesada et al. (1995) demonstrated that such long-distance dispersal events typically result in faster rates of spread as well as accelerating patterns of radial spread. SLF spread rates could increase in this way, and we observed the largest increases in radial spread in the last two years of the study period (2018 and 2019), potentially indicating accelerating spread.

Our estimates of spread rate varied between methods, with the effective range radius method estimating the highest spread rate. The large differences observed between these methods may reflect the discontinuous nature of SLF spread. Measurement of the radial rate of spread of invading organisms was originally envisioned for continuous range expansion (e.g. Skellam 1951) and may not fully capture discontinuous spread such as observed here, which is also reflected in the low variance explained by distance regression spread estimation ($r^2 = 0.098$) (Fig. 4B). The effective range radius approach may provide a more representative measure of spread in this situation as it accounts for both the frequent long-distance dispersals and subsequent spread into the counties between contiguous and non-contiguous counties. For example, a long-distance dispersal event established a SLF population in northern Virginia in 2018, and in 2019, SLF spread to several counties between the eastern Pennsylvania invasion area and the new area in northern Virginia (Fig. 1). The effective range radius approach accounts for the cumulative invaded area as these counties are occupied in subsequent years, whereas boundary displacement does not include those counties in estimates of radial spread. That is, counties closer to the previously invaded area following a long-distance jump are enclosed by the convex hull polygon and do not influence future boundary displacements as they become invaded. However, the effective range radius approach alone may overestimate spread as entire county areas are summed as invaded, while only portions of each county are actually invaded.

Therefore, based on the findings presented here, we estimate the radial spread rate at around 40 km/year based on the average of the two more reliable methods (i.e. effective range radius and boundary displacement). If SLF were allowed to spread without any intervention, spread might be much higher given considerable management efforts are currently targeted to suppress SLF populations and limit movement. For example, active management programs conducted by USDA APHIS include egg scraping, sanitation (i.e. host tree removal) around SLF detections, and insecticide application to tree of heaven designated as trap trees (USDA-APHIS 2018). Insecticide applications were used primarily to kill SLF landing on trap trees and, in later applications, to determine efficacy of insecticides for use on fruit and residential trees (Urban, Calvin, and Hills-Stevenson 2021). Additionally, the State of Pennsylvania's quarantine on movement of goods out of the invaded area is implemented to limit spread of SLF. It

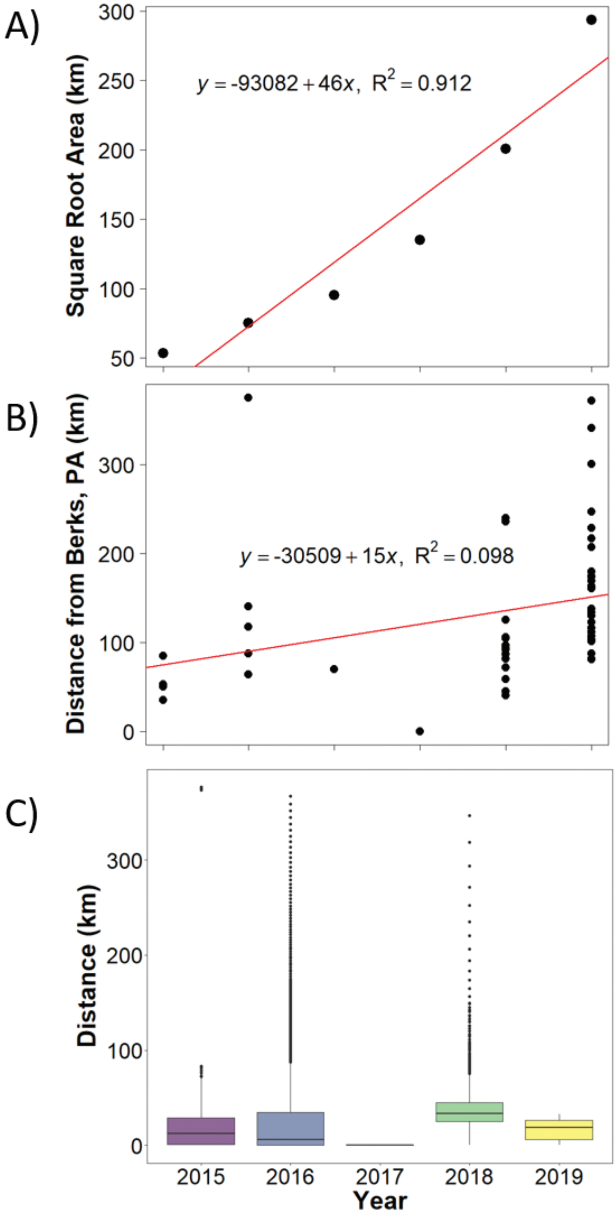

Figure 4. Estimated radial spread rates of spotted lanternfly (SLF) **A** plot of the square root cumulative county area containing SLF establishments divided by π by year of establishment. The slope of the regression is estimated at 46 km per year, providing an estimate of radial spread **B** plot of distance from the centroid of the county with the first SLF detection point (Berks County, PA) by year of establishment. The slope of the regression is estimated at 15 km per year **C** boxplots of boundary displacement distances between years of establishment, with average across all years of 38 km per year and median across all years of 21 km per year.

is also important to note SLF is in the early stages of invasion, and the spread rate may increase as this pest continues to colonize new locations in the USA.

Results of the Cox proportional hazards model suggested that anthropogenic factors, specifically human density, are stronger drivers of SLF spread than forested area or availability of host trees. The role of humans in facilitating spread of invading organisms is a common phenomenon. Known international and domestic pathways of human-mediated spread of tree pests include transportation of pests on live plants (Liebhold 2012) and wood products (e.g. packing materials or movement of firewood) (Yemshanov et al. 2012), though pests can also be transported on non-host materials, such as on stone imports as with SLF. Domestic pathways of human-mediated spread include movement of firewood, transportation via vehicles (e.g. trains, motor vehicles), and "hitchhiking" on travel gear (e.g. hiking gear) and/or pets. Given that SLF lays eggs indiscriminately, human-mediated spread is not limited to host materials. Humans could facilitate the spread of this pest via travel (e.g. automobiles, trains) and movement of both host and non-host materials from an invaded area. Gilbert et al. (2004) came to similar conclusions in their analyses of the horse chestnut leafminer *Cameraria ohridella* Deschka & Dimic (Lepidoptera, Gracillariidae), finding that geographical variation in human population density explained most of the variation in historical spread of this species. Similarly, in an analysis of 79 damaging forest pests, Liebhold et al. (2013) found human population density associated with both spatial proximity and number of invasive forest pests per county across the USA. However, with all such analyses of historical spread, there is always some possibility that statistical associations may be caused in part by more intensive surveying and reporting in more populated areas.

The invasion of tree of heaven in the eastern USA more than 200 years prior to the arrival of SLF may have facilitated the insect's initial establishment, causing an "invasional meltdown" (Feret 1985; Simberloff and Von Holle 1999) in which invasion of one species facilitates the invasion of another. Tree of heaven is the preferred host for SLF and SLF fitness (survival and fecundity) is maximized when feeding on tree of heaven, but this pest can survive and reproduce without access to tree of heaven (Uyi et al. 2020). In addition, SLF's ability to feed on a wide breadth of plant species (more than 70 species) increases the likelihood of the insect encountering a suitable host following dispersal (Dara et al. 2015). The final Cox proportional hazards model did not include a significant effect of tree of heaven abundance on SLF spread, and therefore we found no evidence that this tree species has influenced SLF spread. Surveys for tree of heaven were conducted by many different people including volunteers and residents and thus the resulting data are likely not reflective of true distribution of tree of heaven, despite verification by EDDMapS reviewers. However, given the association of tree of heaven with urban and disturbed environments, it is probable that more accurate tree of heaven distribution data may correlate highly with human population, which emerged as a significant predictor of SLF spread. In addition, as SLF invasion progresses, additional relationships to host trees or other environmental variables may become apparent or the importance of such variables may vary geographically.

Spatial proximity will remain an important predictor in the future spread of this pest, rendering estimation of SLF populations an important step in assessing spread. Current challenges in estimating SLF populations are primarily lack of long-term, systematic population assessment data and difficulties detecting small populations. The SLF-specific dispersal kernel we estimated here provided the best estimates of spatial proximity based on available distribution data but it was limited by the coarse spatial scale of county-level data and the limited temporal replication. We anticipate that as more data are collected on SLF populations, the estimated dispersal kernel could be refined and thus enhance model predictions.

There are a few limitations involved in our study. First, the data used in these analyses consisted of visual surveys that were located based on perceived risk of SLF establishment. These data were not collected in a systematic fashion, and thus there is potential for sampling bias and imperfect detection, e.g. overlooking of individuals. Though work is underway on developing traps to efficiently survey for SLF (Francese et al. 2020), a sensitive SLF-specific trapping system has not yet been widely implemented. The lack of a pest-specific trap increases risk of missed detections in visual assessments, especially for low population densities. Missed detections occur in many invasive species surveillance programs and can lead to underestimation of the extent of species ranges as well as biased estimates of invasion speeds (Guillera-Arroita 2016; Mang et al. 2016). Given these potential biases, we used counties as the unit of analysis, and the estimates of spread rate as well as drivers of local spread at a finer resolution may be different. We also assumed counties with only a single year of SLF detection indicated populations that failed to establish and thus were not detected in future surveys. Failure to establish could be the result of stochastic dynamics or Allee effects, both of which can drive low-density, newly invaded populations to extinction (Liebhold and Tobin 2008). For example, Liebhold and Bascompte (2003) concluded that low density gypsy moth *Lymantria dispar* (L.) populations are likely to reach extinction without intervention, and in their analysis, most of the populations that did go extinct without treatment did so within a year of detection. Where management efforts are in place, failure to establish could also be the result of local eradication efforts. However, there is also a possibility that low-density populations did indeed persist, but due to difficulties in detecting this pest without specific lures or traps, small populations went undetected.

Focusing efforts on assessing populations and on estimating spatial proximity is important in describing and predicting spread of non-native pests. Our findings suggest that SLF has spread from 2014–2020 primarily through local diffusion with less frequent but consistent long-distance dispersal from previously established populations with influence from human populations. Based on the results presented here, we anticipate that SLF will continue to spread in the USA, though management and eradication efforts may effectively reduce population densities, reproductive potential, and ultimately rate of spread. Additional monitoring efforts to prevent and detect long-distance dispersals may prove useful, especially regarding transports of materials from areas with existing SLF populations.

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Supplementary material 1

Figure S1

Authors: Rachel T. Cook

Data type: map

Explanation note: Locations of SLF visual syrveys conducted by the US Animal and Plant Health Inspection Service and Pennsylvania Department of Agriculture.

Link: https://doi.org/10.3897/neobiota.70.67950.suppl1

Supplementary material 2

Figure S2. Distributions of anthropogenic predictor variables used in Cox proportional hazards moder development

Authors: Rachel T. Cook

Data type: maps

Link: https://doi.org/10.3897/neobiota.70.67950.suppl2

Supplementary material 3

Figure S3. Habitat predictor variable distributions

Authors: Rachel T. Cook

Data type: maps

Link: https://doi.org/10.3897/neobiota.70.67950.suppl3

Supplementary material 4

Table S1. Predictor variable summary

Authors: Rachel T. Cook

Data type: statistical data

Link: https://doi.org/10.3897/neobiota.70.67950.suppl4

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RESEARCH ARTICLE

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The potential direct economic impact and private management costs of an invasive alien species: *Xylella fastidiosa* on Lebanese wine grapes

Michel Frem[1,5], Vincenzo Fucilli[2], Franco Nigro[1], Maroun El Moujabber[3], Raied Abou Kubaa[4], Pierfederico La Notte[4], Francesco Bozzo[2], Elia Choueiri[5]

1 *Università degli Studi di Bari – Aldo Moro, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti (Di.S.S.P.A.), Via Amendola 165/A, 70126, Bari, Italy* **2** *Università degli Studi di Bari – Aldo Moro, Dipartimento di Scienze Agro Ambientali e Territoriali (Di.S.A.A.T.), Via Amendola 165/A, 70126, Bari, Italy* **3** *CIHEAM Bari, Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010, Valenzano, Bari, Italy* **4** *Consiglio Nazionale delle Ricerche, Istituto per la Protezione Sostenibile delle Piante, Via Amendola 122/I, 70126, Bari, Italy* **5** *Lebanese Agricultural Research Institute, Department of Plant Protection, Tal Amara, P.O. Box 287, Zahlé, Lebanon*

Corresponding author: Michel Frem (mefrem@lari.gov.lb)

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Abstract

Since its outbreak in 2013 in Italy, the harmful bacterium *Xylella fastidiosa* has continued to spread throughout the Euro-Mediterranean basin and, more recently, in the Middle East region. *Xylella fastidiosa* subsp. *fastidiosa* is the causal agent of Pierce's disease on grapevines. At present, this alien subspecies has not been reported in Lebanon but if this biological invader was to spread with no cost-effective and sustainable management, it would put Lebanese vineyards at a certain level of risk. In the absence of an *Xylella fastidiosa* subsp. *fastidiosa* outbreak, the gross revenue generated by Lebanese wine growers is estimated as close to US$22 million/year for an average period of 5 years (2015–2019). The potential quantitative economic impacts of an *Xylella fastidiosa* subsp. *fastidiosa* outbreak and particularly, the private control costs have not been assessed yet for this country as well as for others which *Xylella fastidiosa* may invade. Here, we have aimed to estimate the potential direct economic impact on growers' livelihoods and provide the first estimate of the private management costs that a theoretical *Xylella fastidiosa* subsp. *fastidiosa* outbreak in Lebanon would involve. For this purpose, we used a Partial Budget approach at the farm gate. For the country as a whole, we estimated that a hypothetical full spread of *Xylella fastidiosa* subsp. *fastidiosa* on Lebanese wine grapes would lead to maximum potential gross revenue losses of almost US$ 11 million for an average recovery period of

4 years, to around US$ 82.44 million for an average grapevine life span period of 30 years in which infected plants are not replaced at all. The first yearly estimated additional management cost is US$853 per potentially infected hectare. For a recovery period of 4 years, the aggregate estimated additional cost would reach US$2374/ha, while the aggregate net change in profit would be US$-4046/ha. Furthermore, additional work will be needed to estimate the public costs of an *Xylella fastidiosa* subsp. *fastidiosa* outbreak in Lebanon. The observed costs in this study support the concerned policy makers and stakeholders to implement a set of reduction management options against *Xylella fastidiosa* subsp. *fastidiosa* at both national and wine growers' levels. This re-emerging alien biota should not be neglected in this country. This understanding of the potential direct economic impact of *Xylella fastidiosa* subsp. *fastidiosa* and the private management costs can also benefit further larger-scale studies covering other potential infection areas and plant hosts.

Keywords
Alien species, biological invasion impact, crop protection, economic impact, partial budget, pest outbreak, pest risk analysis, *Xylella fastidiosa*

Introduction

The biological invasion of alien species is increased by global trade, climate change and economic activities through the transport of humans and plants (McDermott 2015; Chapman et al. 2017; Pratt et al. 2017), and has the potential to cause direct and indirect market, and non-market impacts. The global costs of invasive insects have been determined at a "minimum of US$70.0 billion and more than US$6.9 billion per year for goods and services as well as for human health" respectively (Bradshaw et al. 2016). Further, the economic damages induced by invasive species were estimated for at least close to US$1.3 trillion across the world (Zenni et al. 2021).

Xylella fastidiosa[1], an aerobic gram-negative endophyte bacterium in the Xanthomonadaceae family (Wells et al. 1987), is a good example of this biological invasion process. *Xylella fastidiosa* (Fig. 1) is recognized as a very high-risk pathogen due to: (i) its latency period in many plant species, which favors its conservation and diffusion, (ii) efficient transmission by numerous xylem feeding insect-vectors (spittlebugs, sharpshooters, and/or leafhoppers), (iii) adaptability to varied climatic conditions, (iv) polyphagia, (v) severity of symptoms (common symptoms are leaf scorch, burnt edges of older leaves, stem yellowing and dieback), and (vi) its serious economic, social and ecological impacts (Henneberger et al. 2004; European Food Safety Authority 2015, 2018, 2019). *Xylella fastidiosa* has been a major concern worldwide due to its potential transmission through plant materials across borders and because it is the causal agent for the worldwide spread of many plants diseases (Mette et al. 2019). Furthermore, due to the lack of efficient airport inspection procedures and phytosanitary regulations in

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[1] An overview on the biology and ecology of *Xylella fastidiosa*, its distribution worldwide as well as its private management control is stressed in the Suppl. material 1 *Xylella fastidiosa*: a bio-ecology review of a re-emerging alien biota. The link https://gd.eppo.int/taxon/XYLEFA/distribution would grasp the extent of invasions by *Xylella fastidiosa* worldwide as also outlined in the Suppl. material 1: Table S1.

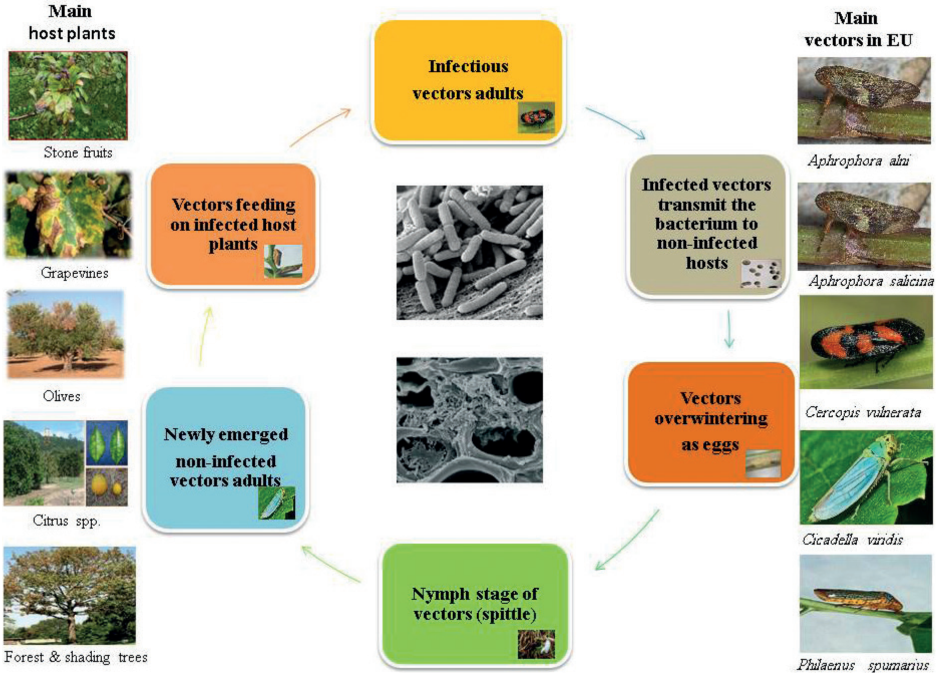

Figure 1. Overview of the life cycle of *Xylella fastidiosa*, its main host plants and vectors in Europe.

the Middle East and North Africa (MENA) region, humans can act as dynamic vectors transmitting potentially *Xylella fastidiosa*-infected planting materials or any infrastructure product carrying insect vectors. Therefore, there is a continual risk of spreading *Xylella fastidiosa* across the world, which has led to recent serious outbreaks. Consequently, countries classified in the high-risk zone should constantly perform specialized pest risk analysis (PRA), implement preventive phytosanitary measures, and focus on enhancing inspection systems, while raising awareness about *Xylella fastidiosa* in order to reduce its economic and ecological impacts (Castrignanò et al. 2020; Frem et al. 2020) and ensure its early detection in asymptomatic plants and vectors (Riefolo et al. 2021).

According to the European and Mediterranean Plant Protection Organization (EPPO 2015, 2016, 2019a, 2019b) *Xylella fastidiosa* was introduced from its native area in the Americas into Asia and Europe. The first European outbreak of *Xylella fastidiosa* occurred in Italy in 2013 (Saponari et al. 2013), followed by France in 2015 (EPPO 2015), Spain in 2016 (Olmo et al. 2017), Germany in 2016 (EPPO 2016), and Portugal in 2019 (EPPO 2019a). Since 2013, *Xylella fastidiosa* has become one of the most harmful pests of vulnerable economic crops (European Food Safety Authority 2019), mainly olives, grapes, fruit stones and ornamental plants, causing socio-economic and landscape damage, and driving economic assessment of potential outbreaks in new countries, such Lebanon. Frem et al. (2020) predicted that the risk level for *Xylella fastidiosa* entry into Lebanon is medium, although the risk of its establishment in the country is at the highest level in the Middle East and North Africa (MENA) region. In order to prevent entry of *Xylella fastidiosa*, Lebanon has imposed specific quarantine measures (Habib et al. 2016; Choueiri 2017) and its country-level of technical readiness is considered as sufficient with medium entry risk (Cardone et al. 2021). Therefore, the diffusion of *Xylella fastidiosa* would put the Lebanese vineyard landscape at a certain level of risk. At present, the bacterium is absent from the study area but we believe that if it spreads without cost-effective management, the economic, social, cultural and environmental consequences will be negative, and a hypothetical outbreak of *Xylella fastidiosa* subsp. *fastidiosa* in Lebanon would involve significant private (i.e. at farm gate) and public costs. Given this, it is useful to assess the potential economic impact of *Xylella fastidiosa* subsp. *fastidiosa* on the Lebanese grape industry, which produces table grapes (70%) and wine grapes (30%). The most common wine grape varieties grown in Lebanon, including red and white cultivars, are characterized in the Suppl. material 2: Table S2.

In this context, this paper aims to assess the quantitative economic impact of a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak on Lebanese wine growers' livelihoods and, to provide the first estimate of the private management costs that would be involved in tackling this potential invasion. For these purposes, a holistic yield loss model (European Food Safety Authority 2019) within a partial budget conceptual approach (Soliman et al. 2010) will be used as suitable economic methodological tools. The added-value of the present research is twofold. Firstly, the economic impact of *Xylella fastidiosa* subsp. *fastidiosa*, based simultaneously on primary and secondary data, has never been assessed in Lebanon. As such, the lack of economic damages of invasive species (potential or observed costs) in Lebanon (Kourantidou et al. 2021) provides some context to this study. The analysis contained in the present research aims to redress this lack of study and to contribute to the scientific literature on economic impact in PRA. In fact, researches based on primary data are lacking from the literature review for the assessment of the economic impact and costs of management of alien species such as *Xylella fastidiosa* subsp. *fastidiosa*. As such, this research provides a useful referential case study that can also be applied in other areas while *Xylella fastidiosa* may spread. In addition, the global reported costs of invasive microbes, and especially bacteria, are very low compared to other more studied taxonomic groups (Leroy et al. 2021) As such, this observation suggests that microbes may be severely understudied, and so any study in this direction, such as the one presented here, will, secondly, enrich the scientific literature on the economic impact of invasive species.

Methods

Previous researches have stressed that economic tools constitute effective measures to manage invasive species which entail crucial costs in terms of control measures or impact (McDermott 2013; Pratt et al. 2017; Barbet-Massin et al. 2020). A range of methodological techniques can be used to analyze the economic impact of an alien species invasion (Pimentel et al. 2001; Born et al. 2005; Pimentel et al. 2005; Olson 2006; Soliman et al. 2010; De Ros 2015; Pratt et al. 2017). In this paper, we have divided the economic assessment approach into two major steps: (i) the potential direct economic impact on wine growers' livelihoods, and (ii) *Xylella fastidiosa* subsp. *fastidiosa* management costs. As such, the Methods section is structured into three parts as follows: (i) data compilation, (ii) estimation method for potential direct economic impact and, (iii) estimation method for private management costs.

Data compilation

For the potential economic impact of *Xylella fastidiosa* subsp. *fastidiosa* on wine growers' livelihoods, we obtained the correspondent secondary data (area, yield and value of wine grapes) between 2015 and 2019 from the Food and Agriculture Organization, FAOSTAT database (www.fao.org/faostat/en/) and the Ministry of Agriculture (2017). There are no official updated data available relating to the price per ton of wine grapes at farm level. Estimates were therefore obtained from the field survey in the study area. Further, there are no quantitative data on the running production costs and additional costs of a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak at vineyard level in Lebanon (Kourantidou et al. 2021). In order to quantify these costs (Table 1), given the absence of updated reliable primary data concerning the production costs in the country, and the need to construct pre-invasion and post invasion scenarios for *Xylella fastidiosa* subsp. *fastidiosa*, we collected and calculated the concerned costs through a specific field survey questionnaire (Suppl. material 9: Field survey) involving a focus group of 76 representative farmers, who are experts in vineyard production. The questionnaire has three sections. The first section collects information about the key farmer and the work-force in the vineyard farming system in the survey region. The second section collects current technical and financial data about the key grape farm (i.e. cultural practices, cultivars, production, cost of production, etc.) within the context of a normal agricultural situation without an *Xylella fastidiosa* subsp. *fastidiosa* outbreak (i.e. scenario A). The third section aims to estimate the additional costs that could be involved over a recovery period of 4 years in order to cope with a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak (i.e. scenario B). For this purpose, we selected farmers from the western area of the Beqaa Valley (Fig. 2), the central region of Lebanon, where *Xylella fastidiosa* subsp. *fastidiosa* may reasonably be expected to have the greatest potential direct economic impact. The selection of this field survey area was based on: (i)

Table 1. Additional costs that could be involved by a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak based on the literature review of main control strategies at farm gate. The table outlines the principal additional costs that could be involved in a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak over a recovery period of 4 years (2020–2023) required for a vine to become productive after replanting (EFSA 2019).

Type of additional costs	Justification
Labor	Removal and disposal of diseased or dead vines as soon as PD appears in the vineyard, in order to reduce its infection rate. Physical removal of weeds under vine plants. Visual monitoring and inspection of vectors with a sweep net. Spraying of chemicals. Pruning: effective pruning on detection of early symptoms.
Insecticides	To reduce the potential population of *Xylella fastidiosa* subsp. *fastidiosa* vectors (leafhoppers insects), adjacent habitats in areas close to the vineyards must be sprayed, mainly in spring.
Herbicides	Soil management: chemical removal of weeds under vines.
Sticky traps	To monitor or observe the movement of potential *Xylella fastidiosa* subsp. *fastidiosa* vectors.

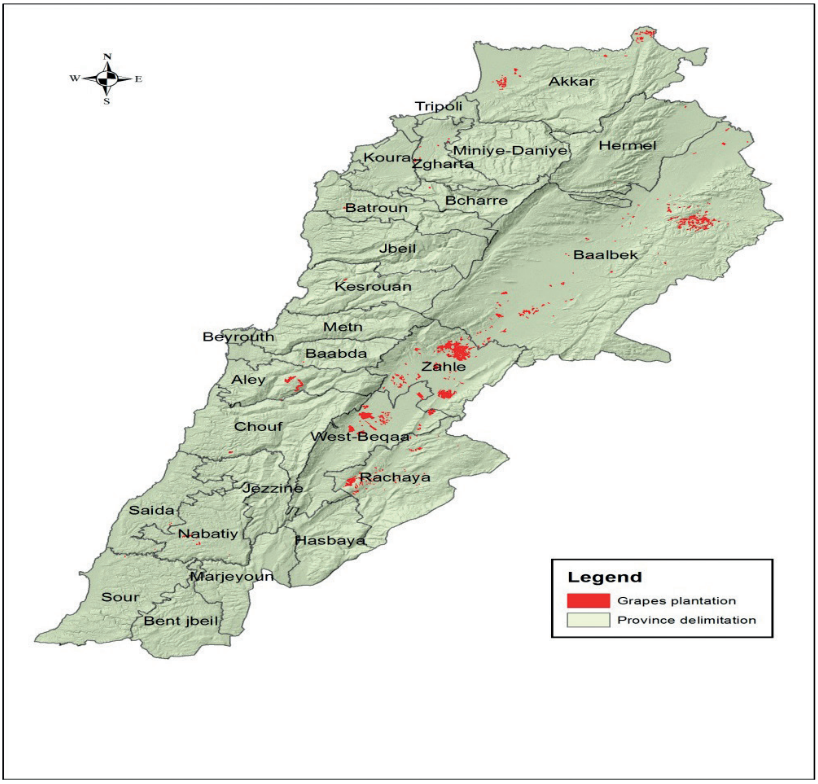

Figure 2. Wine grape plantations in Lebanon based on data from the Ministry of Agriculture (2017). The map highlights the importance of wine grape plantations in Zahlé and West Beqaa that were considered as a field study area in our estimation model.

its suitable quarter summer temperatures (Suppl. material 3: Table S3) for *Xylella fastidiosa* subsp. *fastidiosa* diffusion (around 25–32 °C) as examined by Feil and Purcell (2001), and (ii) its economic importance. In fact, this region is one of Lebanon's most important and oldest commercial wine grape production areas (El Chami and El Moujabber 2014). According to the Ministry of Agriculture (2017), out of 992 farmers (cultivating approximately 3,057 ha of wine grapes) in Lebanon, approximately 600 work in the Beqaa Valley, which has a wine grape surface area of 1,941 hectares, distributed between its main provinces: Baalbek (35%), Zahlé and West Beqaa (65%). At present, 70% of the national wine output is produced mainly by 4 Beqaa Valley wine producers (Chateau Ksara, Chateau Kefraya, Chateau Musar and Chateau Saint Thomas) from grapes grown in this valley with a heritage value. Furthermore, 50% of the region's wine production is exported abroad.

Estimation method for the potential direct economic impact

We estimated the potential gross revenue losses in wine grapes based on yield losses as estimated by EFSA (2019), where the yield loss on wine grapes would oscillate between 1.2% (low impact), 2.1% (medium impact) and 8.1% (high impact). Here, we considered these 3 pressure levels in order to consider the uncertainty of results which would be influenced by winter climate unsuitability (freezing temperatures may reduce proliferation of the bacterium in the production areas, up to 1000 m above sea level), the unknown density of *Xylella fastidiosa* subsp. *fastidiosa* vectors (lack of field studies to confirm their activity, low or high abundance), the short period of effective infection (2 to 3 months starting in June) and cultural practices (vineyard irrigation, insecticide use, heavy pruning of plants, etc.) would give a lower incidence rate.

Estimation method for the private management costs

We based this estimate on the Partial Budget (PB) method (Table 2) as outlined by Soliman et al. (2010) due to: (i) its relevance for the purpose of calculating the additional costs (control costs) and the reduced farmers' incomes (yield loss) of a potential *Xylella fastidiosa* subsp. *fastidiosa* invasion, and (ii) its simplicity, transparency of credibility of results. PB is a basic economic tool that analyzes the changes in costs and revenues due to any unplanned change, such as a pest invasion or management measures (use of insecticides, herbicides, fungicides, weed management, cultural alterations, etc.) in farming systems (MacLeod et al. 2004). Table 2 illustrates PB and Fig. 3 highlights the principal quantitative economic impact assessment methods used in PRA (Soliman et al. 2010)

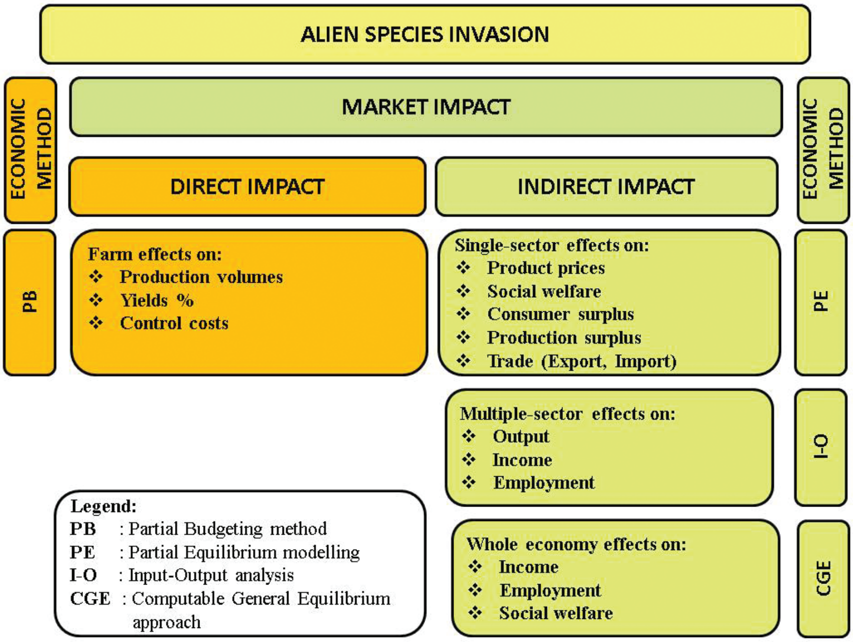

Figure 3. Overview of the direct and indirect market impacts of an alien species invasion in a new location. The diagram highlights the principal quantitative economic impact assessment methods used in pest risk analysis where the Partial Budgeting apapproach was considered in this research. The overview is based on Soliman et al. (2010) and De Ros et al. (2015).

Table 2. Overview of the partial budgeting of *Xylella fastidiosa* subsp. *fastidiosa* invasion on wine grapes, based on the general layout of Soliman et al. (2010). The table aggregates the specific costs and benefits considered in our economic impact model.

Costs	$US	Benefits	$US
Additional costs **(A)**		Additional revenues **(C)**	
Control & protection costs			
Reduced revenues **(B)**		Reduced costs **(D)**	
Yield and/or Quality losses			
Total costs: (A) + (B)		**Total benefits: (C) + (D)**	
Net change in profit: (C) + (D) - (A) - (B)			

and the direct and indirect market impacts of an alien species invasion in a new location (De Ros et al. 2015). According to the Food and Agriculture Organization (FAO 2004), the economic impact assessment of a quarantine pest like *Xylella fastidiosa* subsp. *fastidiosa* is a fundamental component of PRA. PRA aims to outline "economic evidence helping the phytosanitary authority in each country to determine if the studied organism is a pest, whether it should be regulated, and the enhancement of any phytosanitary measures to be undertaken against it" (FAO 2007). Consequently, FAO (2007) has established an international phytosanitary standard (ISPM N°11) focusing on the qualitative (i.e. expert judgement) and quantitative approaches to conduct PRA without giving any preference for the use of either method. The qualitative scheme(s), through focus group expertise, are well structured and cost-effective but appear more subjective because they are based on the opinions of experts and there is a lack of transparency and repeatability.

Results

Economic impact of *Xylella fastidiosa* subsp. *fastidiosa* on wine grapes growers' livelihoods

As an ex-ante situation (i.e. absence of a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak/Scenario A), the gross revenue generated by wine growers is estimated as close to US$22 million, almost 33% of which emanates from the study area. With a potential *Xylella fastidiosa* subsp. *fastidiosa* invasion (Scenario B), the estimated potential annual economic losses to wine grape growers would range from US$ 1.32 to almost 2.75 million (Table 3). Consequently, the upper potential gross revenue losses would be close to US$ 11 million for an average period of 4 years (EFSA 2019) if the infected vines were to be replaced by tolerant/resistant cultivars. If the growers will not be able to uproot and replace their infected plants, the total cultivated wine grapes will not generate any revenue and the upper potential gross revenue losses will amount to approximately US$ 82.44 million for an average grapevine life span of 30 years (EFSA 2019). These values depend on a set of factors: the average area of wine grapes (about 3,082 ha in 2015–2019, i.e. 34% of the total cultivated area of grapes in Lebanon), the average production of wine grapes (about 28,262 tons in the same period), the range of yield loss (low, medium

Table 3. Potential gross revenue losses (US$) of wine grape growers (average based on the period 2015–2019) as retrieved from the FAO database and the range of yield loss as addressed by EFSA (2019).

Period of loss*	Lower impact (Yield loss: 1.2%)	Medium impact (Yield loss: 2.1%)	Upper impact (Yield loss: 8.1%)
1 year	1,322,841	1,503,816	2,748,019
4 years	5,291,364	6,015,264	10,992,076
30 years	39,685,230	45,114,480	82,440,570

*(1) year refers to the first year after a full *Xylella fastidiosa* subsp. *fastidiosa* invasion; (4) years refer to the period of replacement of infected vines and for new vines to bear fruits; (30) years refers to the lifespan of grape vines, if there is no replacement of infected vines. The total wine grape growing area in Lebanon and the price at grower level were assumed to be constant for the entire replacement and lifespan periods.

and high), and the average price ($US734/Ton) of most cultivated Lebanese wine grape cultivars. The average price was estimated from the field survey in which *Chardonnay* has the highest price and *Petit Verdot* the lowest (Suppl. material 4: Table S4).

Private management costs due to *Xylella fastidiosa* subsp. *fastidiosa* spreading

For this purpose, the official currency rate change (US$1 = LBP 1515, year 2019) has been used. The average running cost of wine grape production is US$ 3,824/ha/year prior to an *Xylella fastidiosa* subsp. *fastidiosa* outbreak in the study area (Table 4). In these routine conditions (Scenario A), labor costs were estimated at US$1,269 per ha per year, constituting the highest single cost (33%), followed by the hiring of machinery for plowing and spraying (32%). However, around 3% (US$110/ha per year) of production costs involve by insecticides without specific control of *Xylella fastidiosa* subsp. *fastidiosa* vectors, and almost 7% (US$264/ha per year) involve fungicides. The cost of replacing diseased plants is close to 2%, which may reflect the current good management of vineyards, limiting the impact of diseases.

However, the average additional management costs which could be involved in tackling a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak (Scenario B) at the vineyard level are approximately US$ 853/ha for the first year of the recovery period in which a high infestation rate (40%) and an upper impact on yield loss (8.1%) as shown in Table 5. 8% represents the costs of labor for eradication in terms of removal and disposal of diseased or dead vines as soon as PD appears in the vineyard, in order to control the infection rate. The physical removal of weeds under vine plants accounts for around 4%. The costs of labor for visual monitoring and inspection of vectors with a sweep net, for the spraying of chemicals, and for effective pruning at early symptom onset account for 2%, 5% and 13% of the total additional management costs, respectively. Replantation with resistant plants accounts for the greatest cost, around 33% based on an average price of US$1.70/plant and on an average density of around 2,032 plants/ha for all cultivated cultivars, in which "Merlot" cultivar presents the highest density of around 3,100 plants/ha (Suppl. material 6: Table S6). Most cultivated varieties in Lebanon are very susceptible to PD. However, replantation of varieties less susceptible to PD may affect the quality and the revenues of wine grapes. The use of additional insecticides to reduce the population of *Xylella fastidiosa* subsp. *fastidiosa* vectors (leafhoppers insects) amounts to around 8% of additional costs. It was also assumed that

Table 4. The estimated average running costs of wine grape production (US$/ha/year) in the study area in the context of a normal agricultural situation characterized by the absence of a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak, based on the 2019–2020 production cycle.

Activity	Unit	Average Quantity/ha	Average annual rate	Unit cost	Total (US$/ha)
Replacement of infected plants	Plants	36	1	2	61.20
Plowing hire	Hours	5	1	65	389.57
Sprayer hire	Hours	7	5	23.76	831.68
Total machinery			**1,282.46**		
Chemical fertilizer application	Man-days	3	1	15.18	52.36
Organic fertilizer application	Man-days	5	1	11.22	65.85
Herbicide application	Man-days	3	1	9.24	27.72
Insecticide & fungicide application	Man-days	3	4	9.24	110.85
Irrigation control & maintenance	Man-days	11	2	15.84	370.30
Harvesting	Man-days	26	1	9.24	243.69
Pruning	Man-days	17	1	23.76	397.75
Total labor			**1,268.52**		
Chemical fertilizers – NPK	Kg	220	1	1.00	220.00
Organic fertilizers	Kg	206	1	0.80	164.80
Manure	Tons	2	1	203.30	406.60
Total fertilization			**791.40**		
Mineral oil	Liter	1	3	5.33	15.99
Alpha-cypermethrin	Liter	0.15	3	17.50	6.56
Chlorpyrifos-ethyl	Liter	0.80	3	14.63	38.50
Deltamethrin	Liter	0.20	3	13.00	7.80
Imidachloprid	Liter	0.25	3	11.00	8.25
Indoxacarb	Liter	0.25	4	21.00	21.00
Lambda-cyhalolthrin	Liter	0.20	4	15.00	12.00
Total insecticides					**110.11**
Copper Hydroxide	Kg	1	3	7.00	17.85
Difenoconazole	Liter	0.30	1	19.00	5.70
Micronized sulfur	Kg	4	3	2.00	24.00
Myclobutanil	Liter	0.25	2	33.00	16.50
Penconazole	Kg	0.25	3	36.00	27.00
Sulfur powder	Kg	20	2	4.00	160.00
Trifloxystrobin	Kg	0.13	4	24.00	12.48
Total fungicides					**263.53**
Herbicides	Liter	6	1	6.00	**36.00**
Fuel for pumping	20 L tank	1	6	12.00	**71.00**
Total running costs					**3,823.01**

adjacent habitats would be sprayed in areas close to the vineyards, mainly in spring. Effective active ingredients should be used for successful control of *Xylella fastidiosa* subsp. *fastidiosa* vectors. Regarding the soil management/weeded area, the costs of additional chemicals for the removal of weeds under vine plants constitutes 5% of additional costs, while the use of traps to monitor or observe the movement of potential vectors from surrounding areas into the vineyards constitutes close to 11% of the total additional costs.

Consequently, the gross margin on wine grape production appears to be reduced from 26.33% (i.e. *Xylella fastidiosa* subsp. *fastidiosa* outbreak absent) to around 2% (i.e. *Xylella fastidiosa* subsp. *fastidiosa* outbreak present) in the first year of invasion as described in Table 6 and Table 7. Over a recovery period of 4 years, the aggregate of the additional costs would reach approximately US$2374/ha. For the same period, the aggregate amount of revenues lost due to a yield loss of 8.1% (EFSA 2019) would be about US$1672/ha. As a consequence of a potential *Xylella fastidiosa* subsp. *fastidiosa* invasion, the wine grape farming system (MacLeod et al. 2004) would suffer a loss of US$4,046/ha per 4 years as a net change in profit (Table 2). For the study area, where the total area of wine grapes is about 1,256 ha (41% of the total cultivated area), wine growers would be exposed to a loss of around US$ 5 million, while losses would amount to around US$12.4 million across the country (3,057 ha of wine grapes) for the entire recovery period of 4 years in which the price at grower level was assumed to be constant (Suppl. material 7: S7, Suppl. material 8: Table S8).

Table 5. The estimated average additional management costs of wine grape production (US$/ha/1st year of infection) due to a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak, based on the 2019–2020 production cycle.

Activity	**Additional management costs (US$/ha/1st year of infection)**
Machinery for spraying	99
Labor for uprooting infected plants	66
Labor for mechanical removal of weeds	33
Labor for monitoring of *Xylella fastidiosa* subsp. *fastidiosa* vectors	17
Labor for spraying insecticides	46
Labor for pruning	112
New resistant plants(1)	280
Insecticides	65
Herbicides	45
Traps for *Xylella fastidiosa* subsp. *fastidiosa* vectors	90
Total additional costs (presence of *Xylella fastidiosa* subsp. *fastidiosa* outbreak)	**853**

Table 6. The estimated average gross margin budget (US$/ha/year) in the study area within the context of a normal agricultural situation characterized by the absence of a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak based on the 2019–2020 production cycle.

Component	**(US$/ha/year)**
Total variable costs	3,823
Average yield (Ton/ha)	7.07
Average price (US$/Ton)	734
Gross revenues (US$)	5,189
Gross margin (US$/ha)	1,366
Gross margin (%)	**26.33**

Table 7. The estimated average gross margin budget (US$/ha/1st year of infection), in the study area within the context of an agricultural situation characterized by a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak, based on the 2019–2020 production cycle.

Component	**(US$/ha/1st year of infection)**
Total premium costs (absence of *Xylella fastidiosa* subsp. *fastidiosa* outbreak)	3,823
Total additional costs (presence of *Xylella fastidiosa* subsp. *fastidiosa* outbreak)	853
Average yield (Ton/ha)(1)	6.5
Average price (US$/Ton)	734
Gross revenues (US$)	4,771
Gross margin (US$/ha)	95
Gross margin (%)	**2**

(1) We assumed an upper impact on yield loss (8.1%) in the study area as outlined by EFSA (2019).

Discussion

Main findings at a glance

On average, Lebanon produces 83,125 tons of grapes per year from a harvested area of 9,066 ha, generating a yield of around 9 Tons/Ha in 2015–2019. In 2018, the gross production value of Lebanon's grapes amounted to $US 120 million, representing 4% of the total value of the country's agricultural production (FAO 2020). In the same year, the wine industry produced approximately 8 million bottles (75 cl), and exported around 2,322 tons of grapes, representing 50% of total production with an export value of $US 20.3 million (Banque du Liban et d'Outre Mer 2019). In this paper, we estimated that a hypothetical full spread of *Xylella fastidiosa* subsp. *fastidiosa* on the whole Lebanese wine grapes would lead to maximum potential gross revenue losses of almost US$ 11 million for an average recovery period of 4 years, to around US$ 82.44 million for an average grapevine life span period of 30 years in which infected plants are not replaced at all. Concerning the estimated additional management cost, the amount is US$853 per potentially infected hectare in the first year. For a recovery period of 4 years, the aggregate estimated additional cost would reach US$2374/ha, while the aggregate net change in profit would be US$-4046/ha.

Importance and implications of the findings

The findings explored above provide a clear picture of the potential economic impact and private costs management assessments of a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak on Lebanese wine grapes. Here, we connect the observed results to the existing literature and derive some policy and private implications from our findings. Firstly, our research highlights an economic impact level of a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak in order to manage and identify the control measures to reduce the incidence rate and severity of PD on Lebanese grapevines. Previous studies papers (Soliman et al. 2010; McDermott 2013; Pratt et al. 2017; Barbet-Massin et al. 2020) also assumed the importance of the assessment of the economic impact of invasive species for sustainable policy planning and for the implementation of cost-efficient and environmentally-friendly pest management strategies (Rapicavoli et al. 2018; El Chami et al. 2020). The absence of such a study and the lack of updated and reliable primary data on the costs of production and pest control for Lebanese vineyards is a critical constraint to the design of sustainable management control to mitigate its severe impacts of an invasion pest like *Xylella fastidiosa* subsp. *fastidiosa*, which may affect a total cultivated area of around 3,000 ha of wine grapes in Lebanon. Obviously, wine growers were found ready to adopt an integrated management approach and to put into practice the additional necessary measures to limit *Xylella fastidiosa* subsp. *fastidiosa* damage, which mostly consist of the removal of diseased plants, replantation with resistant/tolerant cultivars, use of appropriate insecticides to control *Xylella fastidiosa* subsp. *fastidiosa* vectors, and soil management. Kyrkou et al. (2018) has summarized the private control strategies against *Xylella fastidiosa* on grapes in 2 categories: (i) prophylactic/preventive measures (i.e. "control of insect-vectors, control of non-vine host plants and vine propagation material, alteration to cropping techniques, breeding PD-resistant/tolerant *Vitis vinifera*, control via avirulent *XYLEFA* strains and control via other beneficial bacteria and fungi") and (ii) therapeutic/curative measures (i.e. "use of bacteriophages of *Xylella fastidiosa* subsp. *fastidiosa*, use of an antagonistic bacterium *Paraburkholderia phytofirmans* strain PsJN, use of natural, antibacterial substances, and use of defense-stimulating compounds"). As the bacterium has high potential to spread in Lebanon through imports of infected host plants or the accidental entry of vectors, the Lebanese policy makers should keep pursuing management strategies to limit *Xylella fastidiosa* epidemics which may remain undetectable up until diseases like PD on grapevines become established. Further, strict policy limitations on the importation, marketing, and transport of plants from countries that are sources of *Xylella fastidiosa* infection are therefore essential in the management program to limit *Xylella fastidiosa* entry and spread. Further public control strategies to *Xylella fastidiosa* subsp. *fastidiosa* outbreak such as monitoring and inspection, certification, screen-house production, and clean (i.e. *Xylella fastidiosa* subsp. *fastidiosa*-free) propagation material (López et al. 2017) should be implemented by the competent local authorities. In addition, local public field trials strategies should be performed to determine the number and timing of spraying and the types of chemical treatments to use in Lebanese vineyards to best control *Xylella fastidiosa* subsp. *fastidiosa* vector populations. In fact, if *Xylella fastidiosa* subsp. *fastidiosa* vectors are not carefully managed, the bacterium has the potential to spread widely in the study area. Further, the lack of early PD notification (intended or unintended by growers or by the local competent authorities) and therefore, any delay in taking immediate action against this plant disease would lead to a spillover into other regions of Lebanon and *Xylella fastidiosa* subsp. *fastidiosa* would spread to other economically vulnerable crops and alternative habitats. Thus, it would probably lead to the expansion of PD across Lebanon, involving further management costs and the potential loss of local and international markets for the country's wines as well as other crops' value chain. Secondly, our analysis shows that the highest private additional costs will be incurred in the first year of infection due to the costs of labor for the removal and purchase of resistant/tolerant plants in order to continue or restart the production of wine grapes within a recovery period of around 4 years. Meanwhile, the analysis of the gross margin highlights that wine grape cultivation is relatively profitable in the study area. Farmers manage their vineyards well in terms of controlling insects, wine grape diseases and weeds. Thirdly, the findings are also important for wine growers and local wineries as the latter are vertically integrated in the farming system, and Lebanon is a net exporter of wines In fact, the potential replacement of the current cultivars of vines, which are mostly susceptible to *Xylella fastidiosa* subsp. *fastidiosa*, or their substitution by PD resistant cultivars, may affect the wine quality and revenues, as well as creating imbalances in supply and demand. Finally, our findings support the concept of multiple "known-on effects" as stressed by Macleod et al. (2004). Nevertheless, the technical measures outlined in this paper would probably not have critical financial consequences for the growers but would generate multiple "known-on effects" in terms of reducing the impacts for local wineries and limiting social effects (unemployment). In the case of an *Xylella fastidiosa* subsp. *fastidiosa* outbreak, local wineries will acquire grapes from other districts to encounter disruption in supply and this will involve additional transactional costs. On the other hand, a hypothetical *Xylella fastidiosa* subsp. *fastidiosa* outbreak may seriously affect the small grape producers, whose livelihoods will decrease in the context of the continuous drastic financial and economical current crisis facing the country.

Comparison of findings with other countries

Although the onset of *Xylella fastidiosa* epidemics is commonly followed by significant economic losses (Rapicavoli et al. 2018), few previous studies have estimated the annual control costs of *Xylella fastidiosa* outbreaks. In USA, annual control costs for Californian oleanders were estimated at US$125.0 million (Henry et al. 1997) and at US$104.0 million for grapevines across 346,000 ha of grapes in California (Tumber et al. 2014; California Department of Food and Agriculture 2018). The total cost of PD attacking grapevines was estimated at almost US$105 million per year (California Department of Food and Agriculture 2018). Using an evaluation model to assess PD impacts, knowing that glassy-winged sharpshooter (GWSS) is its vector in California, the total annual cost of disease control to growers has been estimated as rising by US$189 million per year (cost of replanting and chemical control), with an increase in the grape prices (Alston et al. 2013). This cost excludes the extra US$50 million spent every year on prophylactic phytosanitary measures such as insect control. In Brazil, the annual control costs of *Xylella fastidiosa* on oranges were estimated at US$120.0 million (International Plant Protection Convention 2017). In the absence of effective preventive biosecurity systems, Brazilian orange production has been dramatically affected by *Xylella fastidiosa*. Researchers have estimated an annual loss of US$120 million, corresponding to approximately 6% of total production value in 2003 (Mette et al. 2019). Nevertheless, *Xylella fastidiosa* infection was reduced from 37.6% to 1.3% in 6 years between 2012 and 2018 (Barros 2018), due to the successful implementation of compulsory requirements for importation of certified trees (Almeida and Nunney 2015). Recently, several studies have also been undertaken to estimate the potential economic impact of *Xylella fastidiosa* outbreaks: (i) the costs could vary between US$2.3 billion to US$7.9 billion over 50 years on Australian wine grapes and wineries (Australian Bureau of Agriculture and Resource Economics and Sciences 2018), (ii) annual production losses could reach €5.5 billion over 50 years on European olives (Schneider et al. 2020), and (iii) the values of grapes, olives and citrus spp. production losses have been estimated at around US$10.0 million, US$218.35 million and US$1.0 billion on grapes, citrus *spp*. and olives respectively in nine countries in the MENA region (Cardone et al. 2021). Besides, this pathogen could also affect ecosystem services by damaging rural landscapes, such as Italy's impressive olive orchards, for which the average socio-ecological value of loss has been estimated at between €1,017 and €1,059 per ha (Frem et al. 2021).

Limits of the study

The results explored here underestimate the potential impacts of *Xylella fastidiosa* subsp. *fastidiosa* in Lebanon. Indeed, they represent a fraction of the real potential costs if *Xylella fastidiosa* subsp. *fastidiosa* invades the full territory of the country. Important limits of this research include its assessment of one crop, one region, reliance on direct market impact, missing the indirect market impact and non-market impact of a hypothetical *Xylella fastidiosa* invasion in the country. The main reason of these limitations was the lack of reliable, accurate and updated specific data in the country. Future studies, based on other quantitative economic impact assessment methods (as illustrated in Fig. 2), could use enough resources, generate the requested information and try to assess the indirect market impact of a hypothetical or real *Xylella fastidiosa* invasion at: (i) one single-sector level (effects of *Xylella fastidiosa* on product prices, social welfare, consumer surplus, production surplus, trade), (ii) multiple sectors level (effects of *Xylella fastidiosa* on output, income, employment) and/or, (iii) entire economy level (effects of *Xylella fastidiosa* on income, employment and social welfare). In addition, *Xylella fastidiosa* may also affect seriously other valuable Lebanese economically crops (Citrus spp. stone fruits, olives, etc.) and the entire Lebanese landscape ecosystem (forest and urban trees). As such, the present research, with more specific field surveys, could be extended to include the non-market impact/costs of a potential or real *Xylella fastidiosa* invasion on the ecosystem (provisioning, regulating and cultural services as highlighted in Fig. 2), in the study area and/or in other locations of the country in which the bacterium could lead to serious outbreaks under specific conditions, such as the climate (mainly the quarter summer temperature – Bio10) and habitat (crops, urban ornamental plants and forests) that favor the establishment and spread of *Xylella fastidiosa* (Frem et al. 2020). The discrete choice experiment method would be useful for this purpose.

Conclusion

The potential spread of *Xylella fastidiosa* subsp. *fastidiosa* to new countries like Lebanon is highly likely due to the climatic suitability of grapevine regions. As such, our study illustrates the principal additional management costs that could be involved in tackling a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak at the vineyard level. This research provides important data and valuable information in terms of potential compensation measures to be adopted by policy makers and/or private or international donors if *Xylella fastidiosa* subsp. *fastidiosa* was to spread in Lebanon. On the other hand, in order to reinforce vineyard biosecurity actions, and to encourage early reporting of PD to local authorities for better monitoring and surveillance, we suggest that Lebanese producers potentially affected by *Xylella fastidiosa* subsp. *fastidiosa* should be compensated or receive incentives to offset their losses over the recovery period. Finally, this research could be extended to cover the costs of these types of interventions in order to provide an exhaustive picture of the additional public and private costs of managing a potential *Xylella fastidiosa* subsp. *fastidiosa* outbreak in Lebanon.

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Supplementary material 1

Table S1

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: World distribution of *Xylella fastidiosa*.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl1

Supplementary material 2

Table S2

Authors: Michel Frem, Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: Main characteristics of the most grapevines cultivated in Lebanon. http://www.winepros.org/wine101/wine101.htm

Link: https://doi.org/10.3897/neobiota.70.72280.suppl2

Supplementary material 3

Table S3

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The monthly average temperatures (°C) in Zahlé and West-Beqaa, Lebanon.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl3

Supplementary material 4

Table S4

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The average price (USD/Ton at growers' level) of the most wine grapes cultivars in Lebanon.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl4

Supplementary material 5

Table S5

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The average yield (Ton/ha) of the most wine grapes cultivars in Lebanon.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl5

Supplementary material 6

Table S6

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The average density (plants/ha) of the most wine grapes cultivars in Lebanon.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl6

Supplementary material 7

Table S7

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The study area and countrywide additional management costs in the first year of the recovery period (baseline year: 2020).

Link: https://doi.org/10.3897/neobiota.70.72280.suppl7

Supplementary material 8

Table S8

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: The net changes in profit over a recovery period of 4 years (2020–2023).

Link: https://doi.org/10.3897/neobiota.70.72280.suppl8

Supplementary material 9

Field survey questionnaire

Authors: Michel Frem , Vincenzo Fucilli, Franco Nigro,Maroun El Moujabber, Raied Abou Kubaa, Pierfederico La Notte, Francesco Bozzo, Elia Choueiri

Data type: Occurences

Explanation note: Questionnaire field survey related to the potential economic imapct and private management costs of *Xylella fastidiosa* on Lebanese vineyards.

Link: https://doi.org/10.3897/neobiota.70.72280.suppl9

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RESEARCH ARTICLE

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Invasive predators induce plastic and adaptive responses during embryo development in a threatened frog

Martina Muraro[1], Samuele Romagnoli[1], Benedetta Barzaghi[1],
Mattia Falaschi[1], Raoul Manenti[1], Gentile Francesco Ficetola[1,2]

1 *Department of Environmental Science and Policy, Università degli Studi di Milano, Via Celoria 10, 20133 Milan, Italy* **2** *Univ. Grenoble Alpes, CNRS, Univ. Savoie Mont Blanc, Laboratoire d'Écologie Alpine (LECA), F-38000 Grenoble, France*

Corresponding author: Martina Muraro(marti.muraro93@gmail.com)

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Abstract

Invasive predators can strongly affect native populations. If alien predator pressure is strong enough, it can induce anti-predator responses, including phenotypic plasticity of exposed individuals and local adaptations of impacted populations. Furthermore, maternal investment is an additional pathway that could provide resources and improve performance in the presence of alien predators. We investigated the potential responses to an alien predator crayfish (*Procambarus clarkii)* in a threatened frog (*Rana latastei*) by combining field observations with laboratory measurements of embryo development rate, to assess the importance of parental investment, origin and exposure to the crayfish cues. We detected a strong variation in parental investment amongst frog populations, but this variation was not related to the invasion status of the site of origin, suggesting that mothers did not modulate parental investment in relation to the presence of alien predators. However, cues of the invasive crayfish elicited plastic responses in clutches and tadpoles development: embryos developed faster when exposed to the predator. Furthermore, embryos from invaded sites reached Gosner's development stage 25 faster than those from non-invaded sites. This ontogenetic shift can be interpreted as a local adaptation to the alien predator and suggests that frogs are able to recognise the predatory risk. If these plastic responses and local adaptation are effective escape strategies against the invasive predator, they may improve the persistence of native frog populations.

Keywords

Anti-predator responses, development rate, egg size, parental investment, *Procambarus clarkii*, *Rana latastei*, rapid evolution

Introduction

Biological invasions are a major threat to biodiversity and exert multiple impacts on the ecosystems on a global scale (Bellard et al. 2016, Nentwig et al. 2018). Invasive predators often cause declines and extinctions of native species (Mooney and Cleland 2001). These negative impacts have been often explained by the lack of common evolutionary history, which can hamper predator recognition in native prey and can limit the expression of effective anti-predator responses (Sih et al. 2010). However, there are several mechanisms that allow native species to implement effective responses against invasive predators (e.g. Freeman and Byers 2006; Weis and Sol 2016; Falaschi et al. 2020).

First, prey can display plastic responses to predator selective pressures acting on morphological, life history, physiological and behavioural features (Peacor et al. 2006, Nunes et al. 2014a, Melotto et al. 2021a). Prey generally express phenotypic plasticity when they are able to recognise the alien predator, for instance, because it shares cues with a native predator or is phylogenetically similar to it (Ferrari et al. 2007). Furthermore, aliens can drive strong selective pressures that induce genetic changes in native populations and may determine an evolutionary response of the prey (Cousyn et al. 2001, Nunes et al. 2014a, Ortega et al. 2017, Melotto et al. 2020). If responses to invasive predators are effective, they may increase prey fitness and, ultimately, can allow long-term persistence of native populations. In addition, in some cases, parents can improve the fitness of their offspring through the modulation of parental investment (Cameron and Martin 2000). Parental investment allows modulating the phenotype of offspring on the basis of the conditions experienced by parents (Pick et al. 2019). In many oviparous taxa, egg volume is a major form of parental investment affecting key traits of offspring's fitness, such as survival, morphology, stress tolerance, growth and development rate (Mousseau 1998). Nevertheless, the actual usefulness of a parental investment can be highly context dependent and the increase in parental investment is not necessarily associated with a rise in offspring fitness. Indeed, females may not be able to predict the environment in which their offspring will grow, thus the invested resources would be not appropriate to it (Kaplan 1992). While the selective pressures exerted by invasive species can promote the quick evolution of behavioural and morphological traits limiting exposure to predation (Skelly and Freidenburg 2000, Melotto et al. 2020), there is little information available on the role played by parental investment. Importantly, the effectiveness of parental investments in allowing native prey species persistence remains an open question.

Amphibians are an excellent model system to assess plastic and evolutionary responses and to evaluate the role of parental investment, as they show a broad diversity of phenotypic plasticity, they can rapidly adapt to strong selective pressures and many species are easy to handle under experimental rearing conditions (Kaplan 1998, Relyea 2001, Beebee 2005, McCartney-Melstad and Shaffer 2015). For instance, frogs lay clutches that display a strong variation in number of eggs and egg size and these traits are commonly used to determine the parental investment (Kaplan and King 1997); clutch features are closely related to female body condition and to the environment experienced by mothers (e.g. Dziminski and Ross 2005, Sinsch et al. 2015). Populations of native amphibians exposed to invasive predators often show strong variation in developmental rate. For instance, populations of the Italian agile frog (*Rana latastei*) came into contact with the American red swamp crayfish (*Procambarus clarkii*) in northern Italy approximately 20 years ago (Lo Parrino et al. 2020). The red swamp crayfish exerts a strong predator pressure on amphibians, inasmuch as it is a voracious predator on larvae of amphibians (Cruz et al. 2006, Ficetola et al. 2011b). This invasive crayfish also feeds on amphibian eggs and is able to separate eggs from their protective jelly (Gherardi et al. 2001, Renai and Gherardi 2004). This alien predator is listed amongst the "100 worst" invasive alien species in the world (Cruz et al. 2008, Nentwig et al. 2018). Recent research showed that the Italian agile frog tadpoles are able to metamorphose earlier when exposed to the red swamp crayfish in experimental conditions, especially when belonging to populations already invaded (Melotto et al. 2020). This suggests that both phenotypic plasticity and local adaption can accelerate tadpole development in order to limit exposure to invasive predators (Melotto et al. 2020). However, in invaded populations, the faster development is expected to require higher energetic investments for growth and anti-predator behaviours (Burraco et al. 2020, Melotto et al. 2020). Parental investments might allow parents to partially counteract the constraints posed by the red swamp crayfish. Furthermore, we do not have information on potential responses of early development stages, even though the rate of embryo development can be modulated to reduce predation pressure (Warkentin 2005).

The aim of this study is to test the role of parental investment, phenotypic plasticity and adaptations in the interactions between alien predator and native populations and to evaluate whether parents are able to modulate their investment in response to the presence of an alien predator in *Rana latastei*. We first tested: i) whether variability in parental investment exists amongst frog populations and ii) if this could be related to the invasive crayfish presence as a modulation of maternal investment. Furthermore, iii) we tested whether, under controlled conditions, the rate of embryo development is related to differences in parental investment, whether it is faster in populations invaded by the crayfish (potential local adaptations) or when exposed to the crayfish (potential phenotypic plasticity).

To test these hypotheses, we measured several features of egg clutches to evaluate the variability in parental investment between frog populations invaded and not invaded by the crayfish. Subsequently, we used a common rearing experiment to measure differences in development rates across clutches and tadpoles with different origin, parental investment or exposed/unexposed to the red swamp crayfish. As different climate conditions seem to affect clutch laying in anurans and to avoid differences in parental investment and development time amongst populations living in different climatic conditions (Ficetola and Bernardi 2005), we selected multiple populations from the same altitude and with similar local climate. Our study underlines the importance of phenotypic plasticity and rapid adaptation for anti-predator responses during biological invasions.

Methods

Study system.

The target species of this study is the Italian agile frog (*Rana latastei*), which lives from the sea level up to 500–700 m a.s.l. This frog is endemic of northern Italy and nearby areas and is listed by IUCN as vulnerable due to habitat reduction and fragmentation, pollution and alien species introduction of breeding sites (Schmidt et al. 2020). *Rana latastei* females produce their globular clutches from February to mid-April. Each *Rana latastei* female lay a single egg mass that displays strong variability, with the number of eggs per clutch ranging from 300 to > 2700 eggs (Bernini et al. 2007, Ambrogio and Mezzadri 2018). Clutches are laid in ponds and ditches surrounded by woods and they hatch in 12–15 days, while tadpoles metamorphose in about 3 months. Variation in parental investment (egg size) is known to influence multiple fitness-related traits of larvae, including survival and growth rate (Ficetola and De Bernardi 2009, Ficetola et al. 2011a).

The red swamp crayfish, *Procambarus clarkii,* is native from eastern North America and Mexico, but has been introduced worldwide (except in Australia and Antarctica). This crayfish was introduced in Italy thirty years ago and, since then, its range showed an impressive expansion. Nowadays *Procambarus clarkii* is widespread in Italy and it invaded the study area between 2005 and 2009 (Lo Parrino et al. 2020, Melotto et al. 2020). Landscape-level analyses have shown that the crayfish has strong effects on frog populations, reducing tadpole survival in the wild and affecting the dynamics of population networks (Ficetola et al. 2012, Manenti et al. 2020, Falaschi et al. 2021).

We studied frog populations living in the foothills of the Lombardy Region (north-western Italy). To avoid differences amongst populations living in different microclimatic conditions (Morrison and Hero 2003, Ficetola and Bernardi 2005), we focused on foothill populations living at an altitude of 177–295 m above sea level (a.s.l) within the Monza-Brianza, Como and Lecco Provinces (Suppl. material 1: Table S1). To confirm that the variability amongst clutches in parental investment is unrelated to variation of climatic conditions, we downloaded the ChelsaClim maps at 30-arc second resolution (Karger et al. 2017) and analysed them with QGIS 3.4.13 (https://qgis.org/). We considered two key climatic parameters, known to affect frog fitness and phenology: monthly precipitation and annual mean temperature (Ficetola and Maiorano 2016). The study area is heavily populated, but this region also hosts several fragmented broadleaved forests and wetlands. Within the study area, we sampled eight breeding sites. Each site was represented by a wetland (either a pond or a ditch); all clutches from the same wetland were < 50 m from each other. Four of the eight sites considered are colonised by *Procambarus clarkii*, while four are crayfish-free.

Clutch sampling and measurement of parental investment.

Field activities were performed in February 2020, at the beginning of the breeding season of the Italian agile frog. Sites were monitored daily to collect egg clutches laid during the night before. Newly-laid clutches were photographed in the field to obtain two measures of maternal investment: number of eggs and egg volume. To take pictures, egg masses were removed from the ponds and gently divided in smaller fragments (4 ± 1.5 SD, fragments per clutch) to make eggs individually distinguishable. Clutch fragments were positioned on a white support (mobile table top) wet with the water of the breeding sites. A ruler was placed above the support and photographs were taken with the help of a camera, equipped with a macro lens. The clutch fragments were returned to the breeding sites, except for two small fragments per clutch, which were brought to the laboratory for the common rearing experiment. Overall, we obtained pictures from 50 clutches (total number of pictures processed: 223). We then used ImageJ (Schindelin et al. 2015) to measure the number of eggs and to estimate egg volume (cm^3). To count the number of eggs, the photographs were taken in black and white because the Analyze Particles function requires binarised photographs (Moraga and Pervin 2018). We then used the Threshold function to adjust the photos contrast and the Analyze Particles function to automatically count them and to calculate the average diameter of eggs. Egg volume was then calculated assuming a spherical shape. Finally, the value of total parental investment of each clutch was calculated as total number of eggs × average egg volume.

Development and survival under common rearing conditions

We set up a common rearing experiment to measure differences in development and survival across clutches with different origin, parental investment or exposed/unexposed to the crayfish. We used the same experimental set elaborated by Melotto et al. (2020). Two small fragments (40 ± 12 eggs) from each of the 50 sampled clutches were transported in the laboratory the day after deposition. The two fragments from the same clutch were randomly assigned to one of two treatments: absence of the crayfish or non-lethal presence of the crayfish. The fragments of the clutches were housed in containers and containers were stored in six 70 x 48 cm blocks filled with aged tap water. Clutches belonging to the same blocks were arranged nearby the same central compartment. In half of the blocks, we placed one adult crayfish in the central compartment (predator treatment), while in the remaining blocks, the central compartment was empty (controls). The containers hosting the clutches were separated from the crayfish area by a fine wire mesh that allowed the flow of chemical and visual cues by the crayfish. Control and experimental blocks were maintained under identical outdoor conditions; the three crayfishes were randomly re-assigned to the experimental blocks every 7 days. Half of the water in the blocks was changed weekly and crayfish were fed with flaked fish food and rabbit pellets. The containers were monitored daily to record the timing of hatching (as average time elapsed between the hatching of the first and last tadpoles of the fragment) and the reaching of Gosner's stage 25 (i.e. free-swimming tadpole) (Gosner 1960).

Due to the COVID-19 pandemic, it was not possible to complete the research as originally conceived, because a total local lockdown, starting on 9 March 2020, caused the interruption of laboratory activities. However, despite that, we collected 100 fragments; it was possible to measure hatching time for 42 fragments only (18 collected from sites crayfish-free and 24 colonised by the predator), taken from five ponds. Finally, we determined the time to reach Gosner's stage 25 in 239 larvae: 116 developed with *Procambarus clarkii* non-lethal presence and 123 without crayfish in the rearing experiment.

Data analyses

A linear mixed effects model was used to determine if there was a correlation between number of eggs and egg volume within populations; site of origin was included as the random effect. Adding population as the random factor allowed us to consider differences between populations when analysing variation within populations. We used Pearson's Correlation test to analyse whether there is a covariation between the average number of eggs and the average egg volume of each population. The inclusion of random effect was not necessary when assessing the relationship across populations, as in this case, we only considered one value (average across all the egg masses) for each population.

To assess differences amongst populations in parental investment (egg volume, number of eggs and total parental investment), we used three generalised linear models, one for each parameter describing parental investment, including site of origin as the fixed factor. Subsequently, we used linear mixed effects models (LMMs) to determine the factors related to parental investment across populations (egg volume, number of eggs and total parental investment). Invasion status (invaded/non-invaded by the crayfish), monthly precipitation and annual mean temperature were used as candidate fixed factors with site of origin as the random factor. We then calculated Akaike's Information Criterion (AIC) for all the combinations of fixed independent variables (invasion status and climatic parameters). The model with the lowest AIC value is the one that explains the most variation with the fewest variables and is considered to be the "best model" (Burnham and Anderson 2002). In all models, number of eggs and total investment were log-transformed to improve normality.

LMMs were also used to test factors affecting average hatching time and the time required to reach Gosner's stage 25 (free-swimming tadpole). Average egg volume of the fragment, invasion status and treatment (non-lethal exposure to the crayfish vs. no exposure) were the independent variables. In preliminary tests, we also evaluated statistical interactions between invasion status and treatment. However, these interactions were not significant ($p > 0.3$); consequentially, we excluded them from the analyses. All LLMs included site of origin and rearing block as random factors. We also used the DHARMa R package to assess the residuals of mixed models (Hartig 2021); in all of the analyses, the residuals showed no significant deviations from expectations (for all the models: KS test: $p > 0.43$, dispersion test: $p > 0.79$). We performed all the statistical analyses in R environment, version 3.4.2, (http://www.r-project.org). We used the lme4, lmerTest, car and MuMIn packages for linear mixed models (Kuznetsova et al. 2017) and visreg package (Breheny and Burchett 2017) to generate conditional regression plots. Furthermore, we calculated marginal and conditional R^2 as a measure of effect size in LMMs (Nakagawa and Schielzeth 2013, Johnson 2014).

Results

Parental investment

Fifty newly-laid clutches were collected and photographed to determine the parental investment for each population. We found a strong variation of parental investment across clutches and populations. The number of eggs per clutch ranged from 500 to 2500, while the average egg volume ranged between 0.003 and 0.008 cm^3 (Fig. 1a). The total number of eggs, egg volume and total parental investment showed strong and significant differences amongst frog populations (number of eggs: $F_{7,39} = 6.474$, $p < 0.001$; egg volume: $F_{7,39} = 4.652$, $p < 0.001$; total investment: $F_{7,39} = 6.136$, $p < 0.001$; Figs. 1a, b, c). There was no correlation between number of eggs and egg volume within population ($F_{1,43.6} = -0.247$, $p = 0.812$). Across populations, there was a positive correlation between average number of eggs per clutch and egg volume, but the correlation was not significant at $\alpha = 0.05$ ($r = 0.681$, N = 8, $p = 0.063$).

When we assessed the relationship amongst the three parameters representing parental investment and population features (climate and presence of the crayfish), the null-model always showed lower AIC values, compared to the models including independent variables (Table 1). This suggests that the number of eggs, egg volume and total investment were not related to either climatic parameters or to the presence of the crayfish in the site. Furthermore, none of the independent variables was significantly related to any of the parameters representing maternal investment (Suppl. material 1: Table S2).

Common rearing experiment

The average hatching time of embryos (± SD) was 10.18 ± 0.83 days. Hatching time was not related to the average egg volume (mixed model: $F_{1, 32.5} = 0.029$, $p = 0.867$; Fig. 2c) or to the occurrence of crayfish in the site ($F_{1, 18} = 2.721$, $p = 0.116$, Fig. 2a). Clutches reared with the crayfish in the block hatched faster than controls ($F_{1, 14.6} = 10.786$, $p = 0.005$, Fig. 2b; no significant interaction between invasion status and treatment: $p = 0.227$). The model explained a good amount of variation (marginal $R^2 = 0.17$; conditional $R^2 = 0.69$).

The average time required for reaching Gosner's stage 25 (free-swimming tadpole) (± SD) was 16 ± 1 days. Tadpoles from clutches with smaller egg volumes tended to reach Gosner's stage 25 faster than those with a larger volume one ($F_{1, 24} = 7.138$, $p = 0.013$; Fig. 2f) and we detected significant differences between clutches from invaded vs. non-invaded sites, as tadpoles from invaded sites reached stage 25 significantly earlier ($F_{1, 13.45} = 5.017$, $p = 0.04$; Fig. 2d). The model explained a good amount of variation (marginal $R^2 = 0.47$; conditional $R^2 = 0.78$). However, these results were strongly dependent on the time to reach Gosner's stage 25 of tadpoles hatched from one single clutch fragment with very short time of development. If this fragment was removed from the dataset, the relationship between hatching time and both egg volume and crayfish presence in the ponds of origin become non-significant (egg volume = $F_{1, 23}$ =

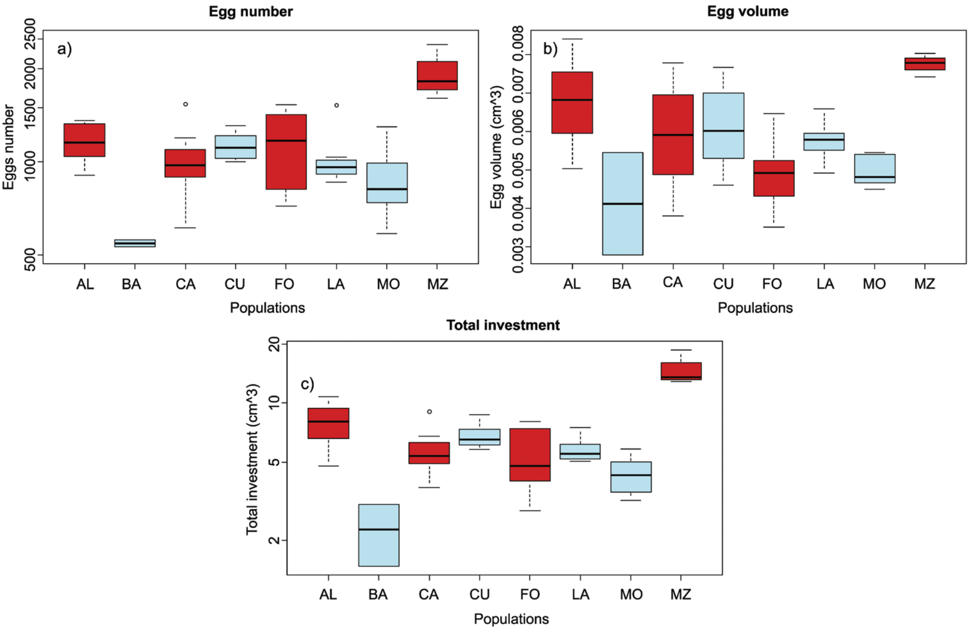

Figure 1. Variability of the three parameters considered describing parental investment amongst populations: **a** egg number **b** egg volume; **c** total investment (i.e. egg number × egg volume). Red boxplots represent crayfish presence in the original ponds, while blue boxplots represent crayfish-free sites. N = 50 clutches. For data analysis, the number of eggs and total investment were logarithm transformed to improve normality; therefore, we show the log-transformed y-axis.

3.442, $p = 0.077$, crayfish presence = $F_{1, 13} = 3.9$, $p = 0.069$). Additionally, in this analysis, tadpoles exposed to the crayfish treatment reached Gosner's stage 25 more quickly than those not exposed to the crayfish ($F_{1, 13.5} = 20.893$, $p < 0.001$; Fig. 2e).

Discussion

Phenotypic plasticity, local adaptation and maternal investment are key mechanisms that can allow withstanding alien predators through the modulation of phenotype. Our study detected strong differences in parental investment across frog populations, even though this variation was unrelated to the presence of the crayfish in the site. We evidenced that tadpole origin and exposure to the crayfish affected the development of frog embryos and larvae, suggesting that plasticity and local adaptations can play a role.

Variation of maternal investment across populations

Very limited information exists about variation of parental investment in *R. latastei*. In literature, just a few counts of the number of eggs are available, with values consistent with our study (Bernini et al. 2004, Ambrogio and Mezzadri 2018). We detected

Table 1. Candidate mixed models assessing the factors related to variation in parental investment across populations. Models are ranked according to their AIC values; models with lower AIC values are the most supported ones by the data. For all the parameters considered, the null model showed the lowest AIC values, suggesting that none of the variables has relevant support. The dependent variables of models are: **a** egg number; **b** egg volume; **c** total investment. The sign of the relationship between parental investment and variables is in parentheses. In Suppl. material 1: Table S1, we also report the significance of the variables in the three mixed models, including all the independent variables.

	AIC	Random factor	Variables
A)Egg number	22.3	Site	–
	23.4	Site	*Procambarus clarkii* (+)
	25.3	Site	Annual mean temperature (+)
	25.7	Site	Monthly precipitation (-)
	27.3	Site	*Procambarus clarkii* (+), Monthly precipitation (+)
	27.8	Site	*Procambarus clarkii* (+), Annual mean temperature (+)
	29.5	Site	Montlhy precipitation (-), Annual mean temperature (+)
	31.4	Site	*Procambarus clarkii* (+), Monthly precipitation (+), Annual mean temperature (+)
B)Egg volume	-479	Site	–
	-465.6	Site	*Procambarus clarkii* (+)
	-464.6	Site	Monthly precipitation (-)
	-463.1	Site	Annual mean temperature (+)
	-450.6	Site	*Procambarus clarkii* (+), Monthly precipitation (-)
	-449.4	Site	Procambarus clarkii (+), Annual mean temperature (+)
	-448.4	Site	Monthly precipitation (-), Annual mean temperature (+)
	-434.5	Site	*Procambarus clarkii* (+), Monthly precipitation (-), Annual mean temperature (-)
C)Total investment	42.9	Site	–
	43.3	Site	*Procambarus clarkii* (+)
	45.3	Site	Monthly precipitation (-)
	45.7	Site	Annual mean temperature (+)
	46.2	Site	*Procambarus clarkii* (+), Monthly precipitation (+)
	47	Site	*Procambarus clarkii* (+), Annual mean temperature (+)
	48.6	Site	Monthly precipitation (-), Annual mean temperature (+)
	49.7	Site	*Procambarus clarkii* (+), Monthly precipitation (+), Annual mean temperature (+)

a very strong variation for both number of eggs and egg volume across females of different populations. Several explanations to this great variability exist, inasmuch as different selective forces and resource availability jointly act on parental investment (Roff 2002). In many cases, the variation in female conditions is a key driver of maternal investment. In amphibians, female body conditions and body size are frequently related to clutch features (Prado and Haddad 2003, Tessa et al. 2009, Chen et al. 2012, Sinsch et al. 2015). In turn, variation in size and conditions can be caused by differences in lifespan, food availability, climatic conditions and other environmental features (e.g. Reim et al. 2006; Roitberg et al. 2013). For instance, previous studies showed that females of the Monza (MZ) population are significantly larger than the ones of other populations from the study area, perhaps because of higher food availability or longevity (Ficetola et al. 2006) and this might allow them to provide better parental investments. Indeed, this hypothesis aligns with our data as Monza females have a significantly higher parental investment for all the parameters considered, if compared to the other populations (Fig. 1).

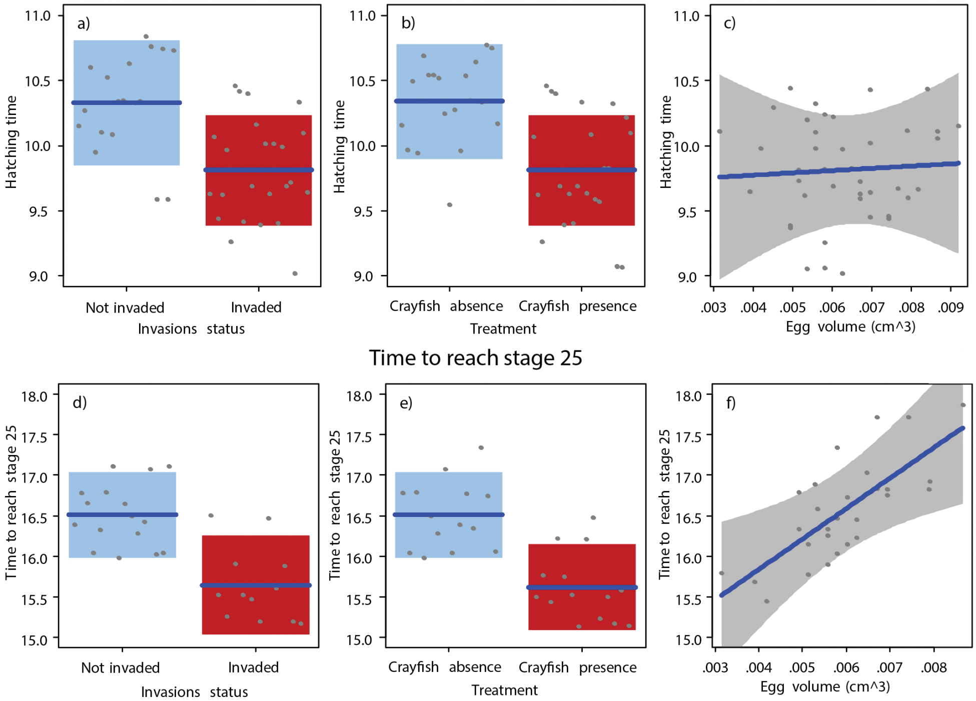

Figure 2. Hatching time and time to reach Gosner's stage 25 of *Rana latastei* in relation to **a–d** the invasion status of populations **b–e** treatment during the rearing experiment and **c–f** egg volume as parameter of parental investment. Red plots represent crayfish presence in the original ponds **a–d** or in the treatment **b, e** and blue plots represent crayfish-free sites **a–d** or the rearing experiment controls (**b, e**). Line shows mean correlation and coloured shaded area shows the 95% confidence interval. N = 42 clutch fragments, N = 239 larvae.

Previous studies showed that differences in parental investment could provide differential fitness advantages under specific environmental conditions in amphibians (e.g. predator pressure, climate, environmental stress) (Dziminski and Ross 2005) and that females can accordingly modulate their investment (Räsänen et al. 2005). For instance, in amphibians, mothers can modify the composition of their egg coats to improve tolerance to acidic conditions in embryos (Shu et al. 2016). We did not detect relationships between maternal investment and climate, as we selected a homogeneous pool of populations to better assess the impact of the alien crayfish, but it will be interesting to assess patterns over a broader climatic gradient. Similarly, variation in maternal investment was unrelated to predator pressure. Other studies demonstrated that amphibians, depending on their life history, modulate parental care in response to geographic differences in climate and in presence of an offspring predator (Delia et al. 2013, Shulte et al. 2020). However, the crayfish has invaded the study area only recently, thus it is possible that the populations have had only a limited time to adapt to this predator (Lo Parrino et al. 2020).

Relationships amongst invasive crayfish, maternal investment and embryo development

Multiple factors affected development rate of *Rana latastei* embryos and tadpoles and crayfish presence in the pond of origin and the non-lethal exposure to the crayfish caused developmental acceleration. Egg provisioning is a key driver of the development rate in frog populations (Dziminski and Ross 2005, Ficetola et al. 2011a). In our study, there were no differences in hatching time related to the average egg volume. Even though hatchlings from clutches with smaller egg volume tended to develop faster than those with a smaller one, this relationship was affected by one single clutch with very short development time. A faster development of embryos hatched from smaller eggs is inconsistent with previous studies (Nussbaum 1985, Berven and Chadra 1988, Dziminski and Ross 2005). In fact, a rapid larval development is often assumed to be positively related to fitness and a larger parental investment is expected to provide resources that can allow embryos to reach faster large size and/or late development stages (Kaplan 1992, Warkentin 1999, Capellán and Nicieza 2006, Ficetola et al. 2011a). However, during the embryo development, the cell cleavage may need more time for larger cells than smaller ones and this could explain the faster development time of small eggs. Further studies, involving analyses of a large sample size, are required to better understand the multifaceted relationships between egg size and time of development.

We observed a significant plastic response in embryos and tadpoles reared in presence of the crayfish; individuals hatched and reached Gosner's stage 25 earlier than those unexposed to the predator. This development acceleration confirms that embryos and larvae can recognise the crayfish cues as a risk. This is the first evidence that *Rana latastei* is able to modify hatching phenology in response to the presence of predators and, thus, the crayfish pressure is strong enough to elicit plasticity in hatching. The co-evolutionary history of species may influence the recognition of a novel predator and, therefore the expression of phenotypic plasticity. Before the crayfish invasion, the Italian agile frog was often syntopic with a native predator that is rather similar to the red swamp crayfish, i.e. the European white-clawed crayfish (*Austropotamobius pallipes*). In turn, the long evolutionary history with a similar native predator can facilitate responses against non-native predators. In fact, a recent study demonstrated that tadpoles of species that co-evolved with the native crayfish are able to recognise the alien crayfish and to better modulate anti-predator strategies when facing the invasive crayfish (Melotto et al. 2021b). Rapid development can be particularly important in the period from hatching to stage 25, when tadpoles are highly vulnerable, given their limited escape ability. The faster development rate can reduce the exposure to this predator, even though tadpoles may suffer costs for other fitness-related traits (Melotto et al. 2020).

Furthermore, we detected differences between colonised and crayfish-free populations in development time. In this case, the effect of *Procambarus clarkii* was only evident after hatching, as tadpoles from invaded sites reached stage 25 significantly earlier, while no differences in hatching time existed. We also acknowledge that the effect of *Procambarus clarkii* after hatching was affected by one single clutch with particularly rapid development, highlighting the importance of additional tests. However, our findings are consistent with the conclusions of Melotto et al. (2020), despite the fact that we tested different populations in different years. This can be interpreted as local adaptation which can limit mortality and suggests that the adaptation to the crayfish occurs in invaded populations. Finally, plasticity and local adaptation often induce coherent phenotypic variation, jointly determining fitness variation across populations (Levis et al. 2018, Stamp and Hadfield 2020). Our study contributes to the growing evidence that invasive predators, as a novel selective pressure, can induce rapid evolutionary changes in native populations (Langklide 2009, Moran and Alexander 2014). Studying potential evolutionary outcomes of native prey is also important to understand the impact of invasive species and to predict potential long-term effects (Sih et al. 2010, Nunes et al. 2014b).

Despite the strong predatory pressure imposed by *Procambarus clarkii*, so far, the total abundance of clutches in invaded populations by the crayfish does not seem to have undergone a significant decrease (Manenti et al. 2020). Frog persistence is certainly due to the immigration of frog individuals from source populations (Manenti et al. 2020, Falaschi et al. 2021), but it is also possible that plastic and adaptive responses to this predator help to counteract the heavy predator pressure, allowing a sufficient number of tadpoles to attain metamorphosis.

In conclusion, we did not observe a significant relationship between variation in parental investment and the occurrence of a major invasive predator, nor did we detect evidence that parental investment improves development rate in this system. Nevertheless, anti-predator strategies, such as phenotypic plasticity or adaptive variations, can help native populations to reduce the impact of an alien predator. So far, most of the studies analysed variation of amphibian performance under laboratory conditions and more studies are needed to understand how these processes act in the wild. Furthermore, in a world where invasive species are increasingly widespread and abundant, continuous monitoring is required to evaluate whether these responses will allow the long-term persistence of native species.

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Supplementary material 1

Geographic coordinates of the monitored sites and the significance of the independent variables in the three parental investment mixed models.

Authors: Martina Muraro, Samuele Romagnoli, Benedetta Barzaghi, Mattia Falaschi, Raoul Manenti, Gentile Francesco Ficetola

Data type: occurrence

Link: https://doi.org/10.3897/neobiota.70.65454.suppl1

Supplementary material 2

Raw data

Authors: Martina Muraro, Samuele Romagnoli, Benedetta Barzaghi, Mattia Falaschi, Raoul Manenti, Gentile Francesco Ficetola

Data type: species data

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Cost-benefit evaluation of management strategies for an invasive amphibian with a stage-structured model

Giovanni Vimercati[1,2], Sarah J. Davies[1], Cang Hui[3,4], John Measey[1]

1 *Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, 7600 South Africa* **2** *Department of Biology, Unit Ecology & Evolution, University of Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland* **3** *Centre for Invasion Biology, Department of Mathematical Sciences, Stellenbosch University, Stellenbosch, 7600 South Africa* **4** *Biodiversity Informatics Unit, African Institute for Mathematical Sciences, Cape Town, 7945 South Africa*

Corresponding author: Giovanni Vimercati (gvimercati@outlook.com)

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Abstract

Management strategies for invasive populations should be designed to maximise efficacy and efficiency, i.e. to accomplish their goals while operating with the least resource consumption. This optimisation is often difficult to achieve in stage-structured populations, because costs, benefits and feasibility of removing individuals may vary with stage. We use a spatially-explicit stage-structured model to assess efficacy of past, present and alternative control strategies for invasive guttural toads, *Sclerophrys gutturalis*, in Cape Town. The strategies involve removal of variable proportions of individuals at different life-history stages and spatial scales. We also quantify the time necessary to implement each strategy as a proxy of financial resources and we correct strategy outcomes by implementation of time to estimate efficiency. We found that the strategy initially pursued in Cape Town, which did not target any specific stage, was less efficient than the present strategy, which prioritises adult removal. The initial strategy was particularly inefficient because it did not reduce the population size despite allocating consistent resources to remove eggs and tadpoles. We also found that such removal might be detrimental when applied at high levels. This counter-intuitive outcome is due to the 'hydra effect': an undesired increase in population size caused by removing individuals before overcompensatory density dependence. Strategies that exclusively remove adults ensure much greater management efficiency than those that also remove eggs and tadpoles. Available management resources should rather be allocated to increase the proportion of adult guttural toads that are removed or the spatial extent at which this removal is pursued.

Keywords

Density dependence, hydra effect, invasive species, management costs, overcompensation, spatially-explicit model

Introduction

Management strategies for invasive populations often aim to eradicate or control the number of invasive individuals in order to minimise their impacts on native species, ecosystems and human activities (Bomford and O'Brien 1995; Robertson et al. 2020). Ideally, these strategies should be designed to maximise both efficacy and efficiency, i.e. to fully accomplish their intended goals while functioning with the least expenditure of resources (Blackwood et al. 2010; Epanchin-Niell and Hastings 2010; Bonneau et al. 2017; Nishimoto et al. 2021). When designing strategies for invasive populations, it is, therefore, desirable to predict not only their absolute outcomes, but also outcomes per unit of resources used (Epanchin-Niell and Hastings 2010; Januchowski-Hartley et al. 2011; Epanchin-Niell and Wilen 2012).

Numerous invasive populations are characterised, at any given time, by cohorts of different life-history stages (also called stage-structured populations; Rodrigues et al. 2015; Hui and Richardson 2017). To maximise reductions in rates of population growth or range expansion, these populations can be eradicated or controlled by allocating a disproportionate management effort towards one or a few specific life-history stages (Ramula et al. 2008; Pichancourt and van Klinken 2012). Deciding on which stage must be prioritised for removal is, however, not always straightforward, because costs and feasibility of removing individuals can vary significantly with their stage in both plants (Taylor and Hastings 2004; Blackwood et al. 2010; Pichancourt and van Klinken 2012) and animals (Buhle et al. 2005; Day et al. 2018). For instance, adult stages are often characterised by fewer individuals, but higher survival rates, than juvenile stages (Lampo and De Leo 1998; Buckley et al. 2005; Govindarajulu et al. 2005; Pardini et al. 2009). Adult and juvenile stages may also be characterised by contrasting behavioural and dispersal capabilities (Govindarajulu et al. 2005; Jongejans et al. 2008; Vimercati et al. 2021) or size and physiology (Beaty and Salice 2013; Green et al. 2014).

Stage-related differences may affect not only the number of individuals that can be detected (detection probability) or removed after detection (intervention success rate), but also how many individuals from different stages can be removed per unit of resource invested (Taylor and Hastings 2004; Mehta et al. 2007; Epanchin-Niell and Hastings 2010). It follows that strategies designed to eradicate or control alien populations often target stages whose individuals are the easiest to detect or remove. This opportunistic approach, however, does not necessarily translate into significant reductions in population size, especially when complex population dynamics exist. For instance, strategies based on the use of electrofishing to control the invasive smallmouth bass, *Micropterus dolomieu*, removed mainly adults, a condition that led to enhance both juvenile recruitment and survival and, consequently, to increase population size (i.e. overcompensation; Weidel et al. 2007; Loppnow and Venturelli 2014). Similarly, applications of herbicides at the rosette stage of the invasive garlic mustard, *Alliaria petiolata*, were largely inefficient in reducing adult abundance, because the initial demographic effects on the rosette density were entirely counterbalanced by marked density dependence survival later in the life-cycle (Pardini et al. 2009). Whenever possible, invasive species population dynamics and information regarding management expenditure should thus be combined to design strategies that maximise outcome while minimising costs.

Invasion dynamics can be reconstructed using a range of mathematical models operating at both individual and population level in accordance with predefined ecological and evolutionary rules (Hastings et al. 2005; Jongejans et al. 2008; Schreiber and Lloyd-Smith 2009; Hui and Richardson 2017). When these models are built to simulate alternative strategies, based on removal of various proportions of individuals at different stages (Buckley et al. 2005; Pardini et al. 2009; Loppnow and Venturelli 2014), strategy outcomes can be corrected by implementation costs to estimate management efficiency (Taylor and Hastings 2004; Epanchin-Niell and Hastings 2010; Epanchin-Niell and Wilen 2012).

In this paper, we assess efficacy and efficiency of alternative management strategies for an invasive population of the guttural toad, *Sclerophrys gutturalis* (Power, 1927), in a peri-urban residential area of Cape Town (Measey et al. 2017). Invasive guttural toads, which were first detected in Cape Town in 2000 (de Villiers 2006), use garden ponds for breeding (Vimercati et al. 2017a, b) and have adaptively responded to the unfamiliar environmental settings of the invasive range (Vimercati et al. 2018, 2019; Madelaire et al. 2020; Barsotti et al. 2021; Mühlenhaupt et al. 2021). In 2010, the City of Cape Town contracted a private company to decrease the population size and limit expansion of the invasive population (Davies et al. 2020a, b) by removing toads from their breeding sites. The breeding sites were mostly located in private properties to which access must be granted by the owners (Vimercati et al. 2017b). In the initial phase of the control operation (2011–2016), adults were disproportionately targeted for removal, although juveniles, metamorphs, tadpoles and eggs were also opportunistically removed (Vimercati et al. 2017a). This strategy was altered in 2017, when data from a preliminary simulation study showed that the removal of eggs and tadpoles reduced the invasive population size to a lesser extent than the removal of adults (Vimercati 2017; Davies et al. 2020b). Consequently, control personnel stopped targeting pre-metamorphic individuals and allocated all management efforts to adult and juvenile removal, a strategy that is currently being pursued (Davies et al. 2020b).

In this study, we explain the rationale behind the decision to change strategies in the control operation of the guttural toad in Cape Town. For management strategies involving the removal of variable proportions of individuals at different life-history stages, efficacy and efficiency were assessed here by the use of a spatially-explicit stage-structured model, which has already been parameterised and validated for this invasive population with field data (Vimercati et al. 2017b). The model has already been used in a recent simulation study to test the efficacy of multiple management strategies based on the removal of a fixed proportion of adult toads at different spatial scales (Vimercati et al. 2017a). This simulation study found that the removal of adults from sites accessible for management did not markedly alter the invasive population size (Vimercati et al. 2017a), because the control team was only able to access a minority of the residential properties located in the area. Hence, we ask here whether a further removal of individuals at other stages, such as juveniles, tadpoles and eggs, may improve management efficacy. As a proxy for financial costs, we also quantify the time necessary to implement alternative management strategies in order to estimate their net efficiency, i.e. their outcome per unit of resource used.

Methods

Model structure

The stage-structured model proposed by Vimercati et al. (2017b) uses a set of integrodifference equations to simulate the spatial dynamics of the invasive guttural toad population in Cape Town within a network of 415 ponds over an area of 27 km^2. The location and size (i.e. small, medium or large) of each pond were obtained through aerial imaging and validated through ground-truthing. In the model, the pond network acts as a meta-population where each pond exchanges individuals with other ponds as a function of a species' dispersal kernel and landscape resistance costs. Within each pond, demographic dynamics are simulated across five life-history stages (i.e. adults, juveniles, metamorphs, tadpoles and eggs). Within the pond network, dispersal dynamics are simulated across two life-history stages (i.e. adults and juveniles). The model realistically captures the life-cycle and invasion dynamics of the guttural toad in Cape Town. Density-independent traits (e.g. adult survival rate and clutch size), density-dependent traits (e.g. tadpole survival rate), detailed integrodifference equations and descriptions of the model are presented in Vimercati et al. (2017b) and summarised in Suppl. material 1.

In brief, an average of 13000 eggs are laid twice a year by each female from late spring (October-November) to late summer (February), with the probability for females to lay eggs in a pond that varies with pond size (Vimercati et al. 2017b). Tadpoles hatch from eggs in one week assuming a constant survival rate (0.7 per individual) and methamorphose in 4–5 weeks as a function of their density in the pond. Over-wintering metamorphs emerge the next spring as juveniles with a probability that varies with their density at the pond edge. Juveniles survive and mature into adults in one year assuming constant rates (0.2 and 0.25 per individual), while adults also survive at a constant rate (0.6 per individual). Each year, fixed proportions of juveniles (0.34) and adults (0.2) disperse across the pond network.

As the population size is reasonably large, demographic stochasticity can be safely ignored; the peri-urban environment has further reduced any effects from environmental fluctuation and uncertainty. First, invasive guttural toads in Cape Town use only permanent, mainly artificial, ponds, thus justifying the assumption that the pond network does not change over time. Second, given the small spatial scale of the invaded area, the climate can be considered homogeneous across the whole pond network, while the landscape structure has not been altered since the first introduction of the species in Cape Town (Vimercati et al. 2017b). Consequently, all life-history traits are set to constant values and landscape features and resistance costs are modelled deterministically. In addition, the model results are robust to changes in the values of most life-history traits, except for changes in the juvenile and adult survival rates which were estimated according to studies on similar species (Vimercati et al. 2017b). The model proceeds for 30 time-steps to simulate 30 years of annual population dynamics, from 2001 (i.e. when the species was first recorded in a single pond of Cape Town) to 2030.

Management simulation

The model structure allows the alteration of mortality rate of any stage at any point in space (different ponds) and time (different years). As a consequence, this model can be used to test alternative management strategies based on different rates of removal across stages (Vimercati et al. 2017a). In accordance with the study conducted by Vimercati et al. (2017a), management strategies were simulated to start in 2011 (i.e. the actual starting year of the management actions in Cape Town) and to end in late 2020. This interval had been chosen to explore the degree to which the alien population can recover in a ten-year period (2021–2030) after management.

First, we designed a management strategy named "initial removal", which realistically simulates removal of the guttural toad in Cape Town from accessible ponds as pursued from 2011 to 2016 by the implementation team (i.e. 128 ponds, see also Vimercati et al. 2017a). As this strategy was implemented in Cape Town without preferentially removing any specific stage (Davies et al. 2020a), we assume, for simplicity, that the proportion of individuals removed across different stages emerges from the interplay between implementers' removal capacity and spatial and temporal occurrence of each stage class in and around the pond. For instance, the proportion of tadpoles that can be removed is expected to be low (0.25), because tadpoles are difficult to detect and capture (e.g. by netting) and they stay in the pond for only 4–5 weeks before metamorphosing. Conversely, the proportion of adults removed from a pond should be high (0.8), because individuals, at this stage, are relatively easy to detect, given their large body size (females) and advertisement calls (males), and they also congregate in or around the pond during the breeding season. The proportions of individuals that are removed for each stage class, according to the "initial removal" strategy and their rationale, are reported in Table 1. Although these proportions may not be exact, field survey, field data and consultation with implementers showed they are realistic (see last column in Table 1 for detailed explanations).

Second, we test a management strategy named "adult removal", which simulates the exclusive removal of adults from ponds accessible to the implementation team. This strategy is currently being pursued in Cape Town, shares with the "initial removal" strategy the proportion of adults removed from each pond (0.8), but differs in that no other stages are targeted for removal. To test whether individuals at early life-history stages should be prioritised over adults, we additionally simulated a third "pre-metamorphic removal" strategy, which is based on the exclusive removal of the same proportion of eggs and tadpoles (0.8) from accessible ponds. We also simulated the hypothetical application of the above three strategies across all ponds (i.e. accessible and not-accessible ponds) in order to explore how management efficacy and efficiency

Table 1. Proportions of guttural toads, *Sclerophrys gutturalis*, removed in Cape Town from ponds accessible by implementers according to the "initial removal" strategy simulated with the stage-structured model. For each stage, the proportion of individuals removed has been estimated by considering: the removal capacity by the implementation team; the spatial and temporal occurrence of the stage in the property visited by the team. Removal proportions have been confirmed by using evidence collected from field data and surveys.

Stage	Proportion of individuals removed	Removal capacity by the implementation team	Spatial occurrence	Temporal occurrence	Evidence from field data and surveys
Adult 	0.8	**High.** Most males and females can be easily detected by the implementation team in and around the pond because of the large body size (Snout to Vent Length [SVL], > 45 mm) and breeding behaviour (e.g. calling in males).	**High.** Most males and females congregate in and around the pond during the reproductive season.	**Medium.** Most males call and stay in and around the pond during the whole reproductive period. Females stay in and around the pond only until the end of egg laying.	Most of the post-metamorphic individuals captured during the management programme were adults (70%). The number of adults removed in a pond at first visit was on average significantly higher than the number of adults detected at second visit.
Juvenile 	0.05	**Low.** Juveniles are difficult to detect because of the small body size (15 < SVL < 45 mm) and the absence of breeding behaviour.	**Low.** Juveniles do not congregate in or around the pond, but are more equally distributed across the invaded area.	**Low.** Juveniles do not congregate in or around the pond during the breeding season.	Only 30% of post-metamorphic individuals captured during the management programme were juveniles. However, the model built in Vimercati et al. (2017b) and other similar models on amphibians (e.g. Beaty and Salice 2013) forecast a number of juveniles between three and ten times higher than the number of adults in the same population. Such a discrepancy between the number of juveniles captured and those that are expected to be present in the population suggests that individuals at this stage are extremely difficult to find.
Metamorph 	0.25	**Low.** Metamorphs are extremely difficult to detect by the implementation team because of the small size (SVL < 15 mm). Additionally, their high density around the pond makes the removal time-consuming.	**High.** Metamorphs stay around the pond edge (within a radius of 5 m) for some weeks after metamorphosis.	**Medium.** Metamorphs stay around the pond for some weeks only after metamorphosis.	Most metamorphs (90%) were detected only during the second part of the breeding season (middle December-February). When metamorphs were detected around a pond, the implementation team succeeded in removing only a minority of them around the pond.
Tadpole 	0.25	**Low.** Tadpoles are extremely difficult to detect and remove by netting in the pond, especially during the night.	**High.** Tadpoles stay in the pond although their removal is more difficult in large ponds.	**Medium.** Tadpoles stay in the pond for 4–5 weeks before metamorphosis.	Tadpoles were removed across the entire breeding season. In most cases, the implementation team could not remove the majority of tadpoles in the pond.
Egg 	0.05	**Low.** Eggs are extremely difficult to detect and remove by netting in the pond, especially during the night.	**High.** Eggs stay in the pond, although their removal is more difficult in large ponds.	**Low.** Eggs stay in the pond for only 5–7 days before hatching.	Eggs were detected and removed much less frequently than tadpoles during the management programme.

vary with restricted access. Analogous to Vimercati et al. (2017a), we additionally simulated a "no removal" strategy, in which the implementation team does not remove any individuals, and a "successful eradication" strategy, in which the removal of most adults, tadpoles and eggs from all ponds leads to a crash in the invasive population. Finally, we quantify to what extent increased efforts in removing eggs and tadpoles improve management efficacy by simulating strategies in which increasing proportions (from 0.6 to 1.0) of post-metamorphic individuals are removed from all ponds.

Efficacy and efficiency assessment

For each management strategy, we estimate efficacy (i.e. the degree to which a strategy accomplishes its goal) and net efficiency (i.e. the efficacy of a strategy corrected by the time [as a proxy for cost spent] on its implementation). As the goal of the initial management programme in Cape Town was to decrease the total number of invasive individuals to zero (i.e. full eradication, Davies et al. 2020b), we use the adult population size obtained just after the end of each simulated strategy (i.e. in 2021) as an inverse proxy for strategy efficacy. Consequently, strategies leading to smaller population sizes are considered more effective than those leading to larger population sizes. The adult population size obtained by simulating a "no removal" strategy (Table 2) is also set as a neutral baseline for efficacy, following Beaty and Salice (2013). As a result, the ratio of the difference between the baseline population size S_0 and the population size obtained by simulating a given strategy (hereafter called S_i) over the baseline population size represents the strategy efficacy E:

$$E = (S_0 - S_i) \tag{1}$$

In other words, E reflects how many invasive individuals would theoretically be removed from the population as a consequence of a given management strategy. For ease of comparison, we also calculate the efficacy in percentage (E%) from the ratio between E and S_0:

$$E\% = (S_0 - S_i) * 100 / S_0 \tag{2}$$

For each strategy, we measured efficiency F as the ratio between E and the strategy implementation cost T expressed in hours. Implementation costs can be estimated in various ways, for instance, by measuring average personnel salary or equipment cost. Here we assume that the management effort invested to control the guttural toad in Cape Town is linearly related to the time spent by the implementation team to remove the toads. This assumption is supported by the observation that the management of guttural toads is done manually without using expensive equipment, while the total salary costs of the implementation team reflect the time spent for removal. We thus conducted field surveys in 2014, 2015 and 2016 to estimate the time (in hours) spent by a manager to target each stage during the initial strategy of removal. We found that at each visit, 1, 0.25 and 0.5 hours have been, on average, allocated to remove adults and juveniles (T_{aj}), metamorphs (T_m) and tadpoles and eggs (T_{te}), respectively.

The removal of adults and juveniles was more time-consuming than the removal of metamorphs: while adults and juveniles can be detected only through a detailed walking survey of the area around the pond, metamorphs are generally found only within 1 to 5 metres from the pond edge, where they congregate to minimise desiccation risk (Vimercati et al. 2017a). The implementation team was also instructed to dedicate a significant portion of their time to detect adults in order to remove reproductive individuals (Scott Richardson, pers. comm.). We consider time spent to remove adults

Table 2. Proportions of guttural toads, *Sclerophrys gutturalis*, removed from each pond according to the different management strategies simulated with a stage-structured model. For each simulated strategy, number of ponds in which the removal is performed, rationale and total time necessary to perform the removal in one year (T) are reported. Please note that the "initial removal" strategy describes the ongoing management of the invasive population in Cape Town (see Table 1), whereas the other strategies describe alternative fictional strategies that could have been implemented.

Management strategy simulated through the stage-structured model described in Vimercati et al. (2017b)	**Proportion of individuals removed from each stage in the simulated strategy**					**Number of ponds visited by the implementation team (N_p) / Total number of ponds in the area**	**Rationale behind the simulated strategy**	**Estimated total yearly time T (in hours) spent by the implementation team to remove individuals from different stages while visiting properties, as expressed in the formula (3): $(T_{aj} + T_m + T_{te}) \times 2 \times N_p = T$**
	Ad.	**Juv.**	**Met.**	**Tad.**	**Eggs**			
"Initial removal"	0.8	0.05	0.25	0.25	0.05	128/415	Estimate of the initial management strategy in which the implementation team remove individuals at different stages from accessible ponds (See also Table 1)	(1 + 0.25 + 0.5) × 2 × 128 = 448
"Adult removal" (current strategy)	0.8	0	0	0	0	128/415	Fictional management strategy in which the implementation team removes only adults from accessible ponds	(1 + 0 + 0) × 2 × 128 = 256
"Pre-metamorphic removal"	0	0	0	0.8	0.8	128/415	Fictional management strategy in which the implementation team removes only eggs and tadpoles from accessible ponds	(0 + 0 + 1.5) × 2 × 128 = 384
"Initial removal in all ponds"	0.8	0.05	0.25	0.25	0.05	415/415	Estimated initial management strategy in which the implementation team removes individuals at different stages from all ponds	(1 + 0.25 + 0.5) × 2 × 415 = 1453
"Adult removal in all ponds"	0.8	0	0	0	0	415/415	Fictional management strategy in which the implementation team removes only adults from all ponds	(1 + 0 + 0) × 2 × 415 = 830
"Pre-metamorphic removal in all ponds"	0	0	0	0.8	0.8	415/415	Fictional management strategy in which the implementation team removes only eggs and tadpoles from all ponds	(0 + 0 + 1.5) × 2× 415 = 1245
"No removal"	0	0	0	0	0	0/415	Fictional strategy in which the implementation team does not remove any individual	(0 + 0 + 0) × 0 × 0 = 0
"Successful eradication"	0.95	0	0	0.8	0.8	415/415	Fictional management strategy in which the implementation team removes most adults, eggs and tadpoles from all ponds	(2 + 0 + 1.5) × 2 × 415 = 2905

and juveniles as a single unit (T_{aj}), as our survey showed that the implementation team usually captures invasive individuals from both these stages within the same area and interval of time. An analogous assumption was made for tadpoles and eggs, which are simultaneously removed from the pond by sweep-netting. The total yearly time spent to remove individuals from different classes (T) is, therefore, obtained according to the following the formula:

$$T = (T_{aj} + T_m + T_{te}) \times N_p \times 2 \tag{3}$$

where the time spent each night to remove individuals across different stages in a single property is multiplied by the number of properties that can be visited (N_p) in one year and by two, which is the average number of properties visited each night. The limited number of properties that can be visited each night by the team (i.e. two properties) is due to the necessity to remove toads when they are mostly active (i.e. within three-four hours after sunset) and the obligation to gain access to a private property at a time that suits the owner (e.g. no later than midnight). As the guttural toad management programme employed only one team to remove toads in 2014, 2015 and 2016, all calculations are based on a single team visiting properties in the evening.

Results

The removal of most adult toads (80%) from accessible ponds ("adult removal" strategy) currently pursued in Cape Town is as effective as the initial strategy (Table 3, Fig. 1), in which adult removal was extended by an additional removal of juveniles, metamorphs, tadpoles and eggs (Table 3, Fig. 1, Suppl. material 2). Moreover, the "adult removal" strategy can be implemented at a lower cost (by 43% of hours spent for removal) than the initial strategy (Table 3, Suppl. material 2). Very similar results are obtained by simulating the application of the same two strategies across all ponds (Fig. 1), with the unique removal of adults that is almost twice as efficient as the initial mode of removal (Table 3, Suppl. material 2). Simultaneously removing individuals at early and late stages (e.g. adults and tadpoles) therefore seems inefficient, because such an intervention prolongs the time spent in each property by the implementation team without providing a commensurate decrease in the guttural toad population size. Intriguingly, our results also show that, when the removal of pre-metamorphic individuals is executed without removing adults in the same ponds ("pre-metamorphic removal" strategy), such a strategy increases the total number of adults in the population (Table 3, Suppl. material 2). Any additional increase in the proportion of eggs

Table 3. Population size at the end of management, efficacy, efficacy in percentage, cost T (in hours) and efficiency obtained by simulating different strategies with a stage-structured model for the invasive population of guttural toad, *Sclerophrys gutturalis*, in Cape Town. Note that the "initial removal" and "adult removal" strategies describe, respectively, the initial strategy (2011–2016, Table 1) and the ongoing strategy (2017-) implemented in Cape Town, whereas the other strategies describe alternative fictional strategies that could have been implemented. In bold, strategies that lead to counter-effective results, i.e. an increase in the population size at the end of management.

Strategy	Population size at the end of management (2011)	Strategy efficacy E, as expressed in the formula (1)	Strategy efficacy E%, as expressed in the formula (2)	Strategy Implementation cost T expressed in hours (as reported in Table 1)	Strategy efficiency F expressed as the ratio between E and T
"No removal"	2973	–	0	0	–
"Initial removal"	2162	811	27%	448	1.81
"Adult removal"	2197	776	26%	256	3.03
"Pre-metamorphic removal"	**3318**	**- 345**	**Counter-effective**	**384**	**Counter-effective**
"Initial removal in all ponds"	494	2479	83%	1453	1.71
"Adult removal in all ponds"	465	2508	84%	830	3.02
"Pre-metamorphic removal in all ponds"	**3897**	**- 924**	**Counter-effective**	**1245**	**Counter-effective**
"Successful eradication"	0	2973	100%	2905	1.02

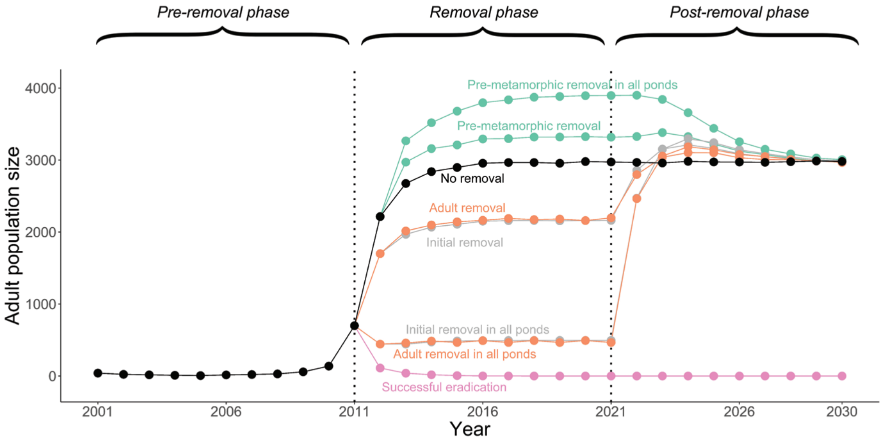

Figure 1. Population size of invasive toads estimated by a stage-structured model simulating alternative management strategies. Adult population size of invasive guttural toads, *Sclerophrys gutturalis*, in Cape Town estimated by a stage-structured model that simulates potential management strategies, as listed in Table 2. Colours (blue, red, grey and purple) indicate removal strategies that are hypothetically carried out by removing different age classes at contrasting spatial scales (accessible ponds vs. all ponds). Black indicates a no-removal scenario. Management was simulated to start in 2011 and to be interrupted in late 2020 (removal phase), after which the model simulating the invasive population would be allowed to run for a further 10 years until 2030. Estimated population size of each fictional management strategy is reported in Suppl. material 2.

and tadpoles removed from the ponds results in a further increase in the total population size (Fig. 2), whereas the full eradication of the population is achieved only by removing all eggs and tadpoles from all ponds. According to our model, the successful eradication of the invasive population could also be achieved by causing a population crash through the removal of almost all adults and most pre-metamorphic individuals from all ponds (Table 3, Fig. 1). The implementation cost of this management effort is estimated to be as much as six times more expensive than that of the initial management strategy (2905 hrs vs. 448 hrs, Table 3) and 11 times more expensive than the current strategy of adult removal (256 hrs).

Discussion

We found that the efficiency of the initial strategy adopted in Cape Town to control the guttural toad was impaired by the removal of eggs and tadpoles; their removal did not noticeably affect the population demography (Fig. 1), but rather subtracted resources (i.e. time) from other modes of removal (e.g. of adult toads). In other words, pre-metamorphic removal did not provide any significant demographic benefit (Table 3)

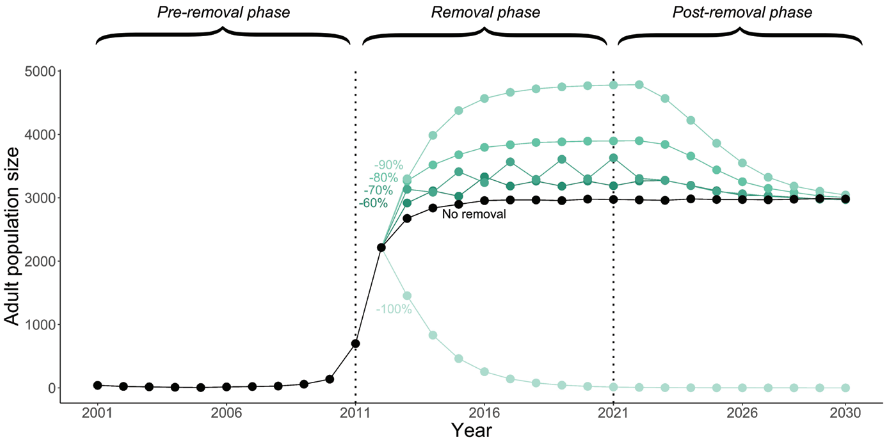

Figure 2. Population size of invasive toads estimated by a stage-structured model simulating different removal proportions of pre-metamorphic individuals. Adult population size of invasive guttural toads, *Sclerophrys gutturalis*, in Cape Town estimated by a stage-structured model that simulates different removal proportions of pre-metamorphic individuals (eggs and tadpoles). Colours indicate different proportions of removal expressed in percentage. Black indicates a no-removal scenario. Management was simulated to start in 2011 and to be interrupted in late 2020 (removal phase), after which the model simulating the invasive population would be allowed to run for a further 10 years until 2030.

and might have even been detrimental when applied with increasing intensity (Fig. 2). The partial removal of pre-metamorphic guttural toads as initially pursued was suboptimal, while the strategy, currently implemented, ensures a much greater management efficiency (Table 1), albeit without leading to eradication (Davies et al. 2020b).

The counter-intuitive observation that a sustained removal of eggs and tadpoles may increase, rather than decrease, the adult population size can be explained by the occurrence of the 'hydra effect'; i.e. "the phenomenon of a population increasing in response to an increase in its per-capita mortality rate" (Abrams 2009). The hydra effect, also defined in some cases as overcompensation (Zipkin et al. 2009; Loopnow and Venturelli 2014; Schröder et al. 2014), has been detected in both structured and unstructured population models as well as in empirical studies (Govindarajulu et al. 2005; Zipkin et al. 2008; Hilker and Liz 2013; Schröder et al. 2014; McIntire and Juliano 2018). The hydra effect may be due to various mechanisms, such as altered patterns of demographic fluctuations (e.g. due to non-linear functional responses), reductions in resource exploitation rates from predators (e.g. due to prey switching from adaptive foraging) and temporal separation of mortality and density dependence (Abrams 2009). We advance that the last of these factors may explain why, in our model, simulated removals of eggs and tadpoles led to the occurrence of the hydra effect. In accordance with other stage-structured models on amphibians (Lampo and De Leo 1998; Vonesh and De la Cruz 2002; Govindarajulu et al. 2005), our model explicitly incorporates density-dependent survival at the tadpole and metamorphic stages to simulate population regulatory processes occurring early in the life-cycle (Vimercati et al. 2017a). Such regulatory processes are common in larval and juvenile stages of anuran species, at least under experimental conditions (Wilbur 1977; Patrick et al. 2008; Berven 2009). However, in Cape Town, the removal of eggs performed by the implementation team increases mortality before the animals could reach the tadpole stage. Furthermore, the removal of tadpoles has been simulated as being performed only shortly after the tadpoles hatch from the eggs, i.e. before their survival is regulated by density dependence. As a consequence, the induced mortality caused by the implementation team "precedes and is concentrated in the early part of a strongly density-dependent stage", a condition that has been considered essential for the existence of the hydra effect (Abrams 2009; McIntire and Juliano 2018). A positive effect of mortality preceding density dependence seems quite common in structured populations (Abrams 2009; Pardini et al. 2009; Loppnow and Venturelli 2014; Schröder et al. 2014; McIntire and Juliano 2018) and should be routinely considered in management planning (Zipkin et al. 2009; Turner et al. 2016), for instance, implementing removal only after density-dependent phenomena (but see Hilker and Liz 2013). The existence of overcompensatory density dependence might also explain why, in native amphibians, low or variable survival rates at early life stages (eggs and tadpoles) have only a minor effect on population growth or decline, in comparison with low post-metamorphic survival rates (Vonesh and De la Cruz 2002; Petrovan and Schmidt 2019; Rose et al. 2021).

Since density dependence in tadpoles is also followed by density dependence in metamorphs, our study also shows that this condition promotes a relaxation of the density-dependent bottleneck; as a consequence, a higher equilibrium density is reached (Schröder et al. 2014). Intriguingly, this could also explain why, once the equilibrium population size is reached, we found a significant difference in adult density amongst ponds of a different size (see Suppl. material 2); small ponds were counterintuitively characterised by a higher number of adults than medium and large ponds. A further indication that higher equilibrium population sizes can be reached under the effect of sequential density-dependent processes comes from the number of individuals observed in the ponds at different life stages forecast by our model. During the saturation phase, small ponds are characterised by low numbers of eggs, tadpoles and metamorphs. However, the situation is completely reversed in juveniles, suggesting that metamorphic density-dependent survival occurring at the pond edge has a much more severe regulatory effect in large and medium ponds. This pattern is not observed in the first years of removal, i.e. when the population was not at the equilibrium, therefore limiting the possibility to implement a management strategy during the initial spread that maximises adult removal by targeting ponds with a specific size.

The occurrence of a strong positive mortality effect at the population level implies that management actions to control the guttural toad should target eggs and tadpoles only when it is possible to fully remove them (Fig. 2); for example, by periodically draining a pond (Doubledee et al. 2003; Maret et al. 2006) or using chemicals (Campbell and Krauss 2002; Witmer et al. 2015). Under the assumption that fish predation may have strong effects on anuran population dynamics (Schmidt et al. 2021), controlled introductions of native carnivorous fish in garden ponds was also theoretically contemplated as a potential means to decrease guttural toad population size. An analogous approach, based on the use of a native top predatory fish, the northern pike, *Esox lucius*, to control the invasive American bullfrog, *Lithobates catesbeianus*, has been proposed in Belgium (Louette 2012). The lack of selectivity of these techniques may, however, limit their utilisation in the field because they can cause collateral negative effects on non-target populations of anurans, fish and invertebrates (Maret et al. 2006). Recent studies examined the feasibility of introducing species-specific chemical inhibitors of tadpole development into breeding ponds of invasive cane toads, *Rhinella marina* (Beaty and Salice 2013; Clarke et al. 2016). The degree to which the same technique can be utilised in other populations of invasive toads is currently unknown. In light of our study, however, all the above techniques should be used only when their implementation can completely eliminate pre-metamorphic individuals or when management resources allow removing both the most pre-metamorphic and post-metamorphic individuals from all ponds ("successful eradication", Table 2, 3, Fig. 2).

Multiple studies on amphibians have shown that variations in the survival rate of juveniles and sub-adults may have severe population-level effects (Vonesh and de la Cruz 2002; Beaty and Salice 2013; Petrovan and Schmidt 2019; Rose et al. 2021). Therefore, it has been recently suggested that both adults and juveniles of guttural toads should be simultaneously removed (Davies et al. 2020b). However, here we advocate that juvenile removal should never be pursued at the expense of adult removal to control the guttural toad in Cape Town. We observed a considerable discrepancy between the juvenile/adult ratio estimated by captures and the ratio forecast by the model (1:3 and 10:1, respectively). This discrepancy is not surprising, as juvenile amphibians are often more evenly distributed across space and time in comparison with adults which congregate at ponds during breeding periods (Pittman et al. 2014, Table 1). However, we also found that, in our simulation study, an equal removal of 80% juveniles instead of adults from the accessible ponds creates a less severe effect on the population demography (reducing 20% versus 30%, respectively, full data not reported). This contradicts the observation by Govindarajulu et al. (2005) who modelled the management of the invasive American bullfrog and reported the removal of juveniles was more effective than removing an equal number of adults. Adult bullfrogs, however, may cannibalise juveniles and this behaviour was explicitly incorporated in their model. Conversely, toads rarely ingest other anurans (Measey et al. 2015) and this foraging preference makes the demographic impact of such intraspecific interaction negligible in the guttural toad. Given the extremely low capacity to detect and remove juveniles and the limited impact their removal has on adult population size, we do not advocate adopting management strategies, mainly or exclusively, based on the removal of juveniles.

Conclusion

Here, we have shown that the strategy currently adopted to control the invasive guttural toad in Cape Town ensures much greater management efficiency than the strategy initially adopted in 2011. By removing only adults, the implementation team can maximise the reduction of population size without dissipating resources for removal of other stages or causing unwanted consequences, such as those associated with the hydra effect. The management resources, saved by not removing pre-metamorphic individuals, should rather be allocated to increase the proportion of adults that are removed or the spatial scale at which this removal is pursued. Overall, our study demonstrates that simulation models, combining complex population dynamics with management costs and field data, represent valuable tools to guide and improve management decisions for stage-structured invasive populations.

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Supplementary material 1

Supplementary material A

Authors: Giovanni Vimercati, Sarah J. Davies, Cang Hui, John Measey

Data type: Text

Explanation note: Supplementary Material A contains a general description of the stage-structured model used to simulate the population dynamics of guttural toads, *Sclerophrys gutturalis*, in Cape Town.

Link: https://doi.org/10.3897/neobiota.70.72508.suppl1

Supplementary material 2

Supplementary material B

Authors: Giovanni Vimercati, Sarah J. Davies, Cang Hui, John Measey

Data type: Demographic database

Explanation note: Supplementary Material B contains: latitude, longitude, ID and size of the ponds that can be used for breeding by guttural toads, *Sclerophrys gutturalis*, in Cape Town and that have been used in the stage-structured model to reconstruct the guttural toad population dynamics; number of adults occurring in each pond and total adult population size as computed by the stage-structured model simulating different management strategies (Table 2, Fig. 1).

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RESEARCH ARTICLE

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Tools for increasing visual encounter probabilities for invasive species removal: a case study of brown treesnakes

Staci M. Amburgey[1], Amy A. Yackel Adams[2], Beth Gardner[3],
Bjorn Lardner[4,5], Adam J. Knox[2,6], Sarah J. Converse[7]

1 *Washington Cooperative Fish and Wildlife Research Unit, School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat Street, Seattle, WA, 98195, USA* **2** *U.S. Geological Survey, Fort Collins Science Center, 2150 Centre Avenue, Building C, Fort Collins, CO, 80526, USA* **3** *School of Environmental and Forest Sciences, University of Washington, 123D Anderson Hall, Seattle, WA, 98195, USA* **4** *Department of Fish, Wildlife, and Conservation Biology, Colorado State University, Fort Collins, CO, 80523, USA* **5** *Present address: Bokekullsvägen 6C, 27730 Kivik, Sweden* **6** *Present address: Maui Invasive Spaces Committee, P.O. Box 983, Makawao, HI, 96768, USA* **7** *U.S. Geological Survey, Washington Cooperative Fish and Wildlife Research Unit, School of Environmental and Forest Sciences & School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat Street, Seattle, WA, 98195, USA*

Corresponding author: Staci M. Amburgey (sma279@uw.edu)

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Abstract

Early detection and rapid response (EDRR) are essential to identifying and decisively responding to the introduction or spread of an invasive species, thus avoiding population establishment and improving the probability of achieving eradication. However, detection can be challenging at the onset of a species invasion as low population densities can reduce the likelihood of detection and conceal the true extent of the situation until the species is well established. This is doubly challenging if the invading species displays cryptic behavior or is nocturnal, thus further limiting opportunities for its discovery. Survey methods that maximize a searcher's ability to detect an incipient population are therefore critical for successful EDRR. Brown treesnakes (*Boiga irregularis*) on Guåhan are a classic cautionary example of the dangers of not detecting an invasion early on, and the risk of their introduction to other islands within the Marianas, Hawai'i and beyond remains. Nocturnal visual surveys are known to detect brown treesnakes of all sizes and are the primary detection tool used by the Brown Treesnake Rapid Response Team, but detection probability remains low in complex forest habitats. As such, we investigated the use of two potential enhancements to nocturnal visual surveys – a live mouse lure and spray scent attractant – that may create hotspots of increased detection probability during surveys. We found that, while brown treesnake detection probabilities were low for all surveys, visual surveys conducted on transects with live mouse lures resulted in detection probabilities that were 1.3 times higher than on transects without live mouse lures. Conversely, the spray scent attractant did not increase the probability of detecting brown treesnakes compared to transects without scent, and in fact had detection probabilities that were 0.66 times lower, though the reasons for this phenomenon are unclear. Unlike scent attractants, live mouse lures likely provide both visual and olfactory cues that attract brown treesnakes to transects and thus provide more opportunities to detect and capture them. These enhancements were trialed on Guåhan, where prey populations are depressed. It remains unclear whether live mouse lures will be as effective for EDRR applications in prey-rich settings.

Keywords
Detection probability, early detection, Guam, lure, rapid response, spatial capture-recapture

Introduction

In invasive species management, the ability to quickly detect and decisively respond to the introduction or spread of an invasive species is often cited as key to the efficacy and success of eradication (i.e., early detection and rapid response or EDRR; National Invasive Species Council 2003, Hulme 2006). This can be challenging at the onset of a species invasion as low population densities can reduce the likelihood of detection and conceal the true extent of the situation until the invasive species has become established (Yackel Adams et al. 2018). Species that display cryptic behaviors and are nocturnal exacerbate this challenge, thus further limiting opportunities for their incidental discovery. Survey methods that maximize a searcher's ability to detect an incipient population are thus critical for successful EDRR. These methods may include those that supplement direct observations of the species of interest (e.g., eDNA; Dejean et al. 2012) or use attractants that draw in target species (Flaherty et al. 2018), all in the pursuit of creating and deploying an optimal suite of tools for conducting EDRR (Morisette et al. 2019; reviewed in Larson et al. 2020). However, unless field testing is undertaken well in advance of the need to deploy such methods, an emergency response may be delayed and potentially ineffective.

The brown treesnake (*Boiga irregularis*) provides a classic example of the dangers posed by a species characterized by a low detection probability that, in combination with belated concern, resulted in a delayed response to its establishment on Guåhan (in the CHamoru language, known in English as Guam) (Rodda et al. 1992). Brown treesnakes were accidentally introduced to Guåhan in the late 1940s, where they proceeded to decimate the native vertebrate fauna over the next several decades (Savidge 1987; Rodda and Savidge 2007). Now, with much of Guåhan's native vertebrate species either declining, locally extirpated, or extinct, a primary objective of brown treesnake management is to prevent the spread of the snake to other islands (Engeman et al. 2018). Due to cultural, recreational, and military pathways of movement and cargo shipments between Guåhan and islands in the Commonwealth of the Northern Marianas (CNMI), Hawai'i, and beyond, there is an ever-present risk of the accidental spread of snakes (Engeman et al. 2018). Cargo inspections and other interdiction efforts were implemented in 1993 (Vice et al. 2005; Perry and Vice 2009) and continue today (Office of Insular Affairs/U.S. Department of the Interior 2020) with success in reducing the spread of snakes (Vice and Vice 2004). Despite efforts, by 2007, there were more than 100 reports of individual brown treesnakes (confirmed and unconfirmed) found on 3 continents and multiple oceanic islands that are thought to have originated on Guåhan (Stanford and Rodda 2007).

To respond to these reports, the U.S. Geological Survey (USGS) created the Brown Treesnake Rapid Response Team (RRT) in 2002. Among its responsibilities, the RRT is an inter-agency and inter-governmental body that serves as an on-call reporting and response service for snake sightings and provides training on the recognition and handling of brown treesnakes to people across the region (Stanford and Rodda 2007). The RRT Coordinator is tasked with establishing the credibility of all reported sightings and, if credibility is established, deploying a team to the location to initiate a search for any individuals. The primary detection tool used by the RRT is nocturnal visual surveys, as that tool has proven more effective in catching snakes of all sizes as compared to any of the available passive (e.g., trapping) methods of detection and capture (Christy et al. 2010). The effectiveness of the RRT was illustrated in October 2020, when they detected brown treesnakes on deployment to the previously snake-free island of Islan Dåno' (known in English as Cocos Island), just south of Guåhan, thereby mobilizing an ongoing EDRR effort (U.S. Geological Survey, 2020). No other incipient populations of brown treesnakes have ever been detected outside of Islan Dåno', due in a large part to the interdiction efforts and the work of the RRT (Yackel Adams et al. 2018, Yackel Adams et al. 2021).

Though visual surveys can be effective in detecting individuals, detection probabilities of brown treesnakes tend to be quite low overall, due to the snakes' use of complex habitat, cryptic behavior and coloration, and nocturnal activity patterns. While direct comparison is challenging as effort level is not easily translatable between methods, detection probabilities of $\hat{p} < 0.15$ (i.e., probability that an individual snake in the effective survey area is encountered on a given night) have been reported for typical surveys using both searching and trapping methods (Christy et al. 2010, Tyrrell et al. 2009). Consequently, a substantial amount of effort is required to infer the absence of an incipient population (Yackel Adams et al. 2018). Therefore, any method that can increase searchers' ability to encounter brown treesnakes would be highly valuable to future rapid response efforts.

As a potential tool to maximize detection probabilities during visual surveys, we investigated the use of two potential attractants for use in EDRR: 1) snake traps that contain a protected live mouse lure and 2) a scented spray applied to surveyed transects. Mouse lures can be detected by brown treesnakes from up to 20 m away (Klug et al. 2015), meaning this attractant, by providing both an olfactory and visual cue, may create hotspots for snake detections in the area surrounding traps. Similarly, other olfactory attractants applied to a transect may also entice and create a "path" for snakes to follow and concentrate around surveyed transects. Solely relying on traps on transects to capture snakes may not be effective (Yackel Adams 2018); for example, Amburgey et al. (2021) obtained 255 camera trap photos of snakes (not necessarily unique individuals) in the vicinity of traps over 45 days, but only 5 snakes were captured in traps. Pairing live mouse lures or spray applications with visual searches may help increase the probability of detecting and capturing a snake, which is especially important in an EDRR context where finding and removing all snakes on the landscape is critical.

Methods and materials

We conducted two field experiments within the Closed Population (CP), a 5-ha (50,000 m^2) fenced area on Andersen Air Force Base in the north of Guåhan. The fence, consisting of a 1.5-m tall, galvanized mesh and chain link wall, had a bulge on both sides about 1.2 m above ground level that eliminated immigration and emigration of snakes in the study area. This fence was also bounded by a 0.5-m concrete footer and vegetation was removed 2 m to either side of the fence to provide a study population of brown treesnakes for investigation of management and population estimation methodologies (Tyrrell et al. 2009, Christy et al. 2010). This area predominantly occurred on coralline limestone with a mix of native and introduced tree species with a canopy height of 5 to 15 m. The composition of trees, shrubs, and herbaceous vines in this area was representative of much of the historically disturbed landscape of the island (Stone 1970). Within the CP were 27 parallel transects cut through the vegetation and spaced 8 m apart. A georeferenced grid cell marker was located every 16 m along each transect, creating a study area of 27 × 13 grid cells (or 351 transect points). This design allowed visual searches to be done on transects with and without experimental treatments in addition to allowing observers to assign snakes to a georeferenced grid cell when captured.

Teams of two observers conducted night-time surveys. Snakes in the CP were part of an ongoing, multi-year (starting in 2004) capture-mark-recapture (CMR) study using unique ventral scale clip patterns and internal passive integrated transponder (PIT) tags. When searchers found a snake, they attempted to scan it without handling to avoid disturbing the individual. If a PIT tag could not be remotely scanned, searchers captured snakes and further checked for a mark or PIT tag or gave a unique mark and PIT tag to previously unmarked animals. In traditional CMR, searchers avoid disturbing traps to avoid deterring animals from being captured; however, as many animals were already marked in the CP and the objective of these surveys was to test the efficacy of EDRR tools, searchers instead focused on checking these areas for snakes.

Because all data were analyzed using a framework that assumes demographic closure of the population (i.e., no immigration, emigration, births, or deaths), we truncated the data for both projects to a two-month timespan. During this two-month period, while demographic closure cannot be guaranteed, there was a low probability of new individuals entering (i.e., being born into or found for the first time) the surveyed population or existing individuals dying. The CP was closed to emigration and immigration due to the two-way barrier surrounding the entire study area.

Live mouse lure

Searchers conducted 25 surveys between February 1 and March 31, 2015. During these surveys, transects either had no traps or live mouse lures (henceforth, no lures) placed on them or had snake traps with live mouse lures (henceforth, lures) placed at all 13 grid-cell markers on a transect. Eleven to 13 transects were surveyed every evening with four to five of these transects having lures. Lures were rotated to new locations every one to three weeks (Fig. 1A). For this design, every other transect (14 total) never had lures placed on them. The other 13 transects alternated between having lures or not depending on the rotation schedule (hence, sometimes these transects were part of the "no lure" treatment and sometimes part of the "lure" treatment). If a snake was captured inside a trap and found during a survey, it was scanned and released by searchers that same night. However, we limited our analysis to snakes found visually by searchers.

Sprayed scent

Searchers conducted 32 surveys between November 1 and December 30, 2016. During these surveys, transects were either unsprayed (henceforth, no scent) or sprayed either in the early evening before the night-time survey (fresh scent) or the previous day (old scent). We distinguished these latter two groups from each other to account for a potential lingering effect of scent. The scent consisted of a mixture of 500 ml fish fertilizer (Alaska Fish Fertilizer) and 14.74 L of water and was sprayed along the entire length of a transect on the ground (1–1.5 feet above the surface) over the course of four minutes to ensure a consistent application rate. The mixture was emitted in a flat, constant spray that resulted in little drift and even application, requiring a little under 14.74 L for three transects-worth. This scent mixture was selected from a pilot study that also tested beef blood and canned tuna mixtures (B. Lardner & A. Knox, *pers. comm.*), with the fish fertilizer eliciting the highest level of brown treesnake activity (as quantified by number of times animals entered and investigated an area with the scent applied). Brown treesnakes are scavengers that will consume a variety of carrion (Savidge 1988), and all the scents tested were readily accessible on island or easily shippable.

On most evenings of the study, nine transects were sprayed with scent and continued to be sprayed daily for three days in a row. The other 18 transects were surveyed but no spraying occurred. On the fourth day, no new spraying occurred but all transects were surveyed. After this 4-day surveying bout, a three-day break occurred after which nine new transects were sprayed (Fig. 1B). On two occasions, spraying was not completed (e.g., due to heavy rain) and only partial surveys were conducted. All transects were eventually sprayed across the study period, with each transect being part of at least three different spraying bouts.

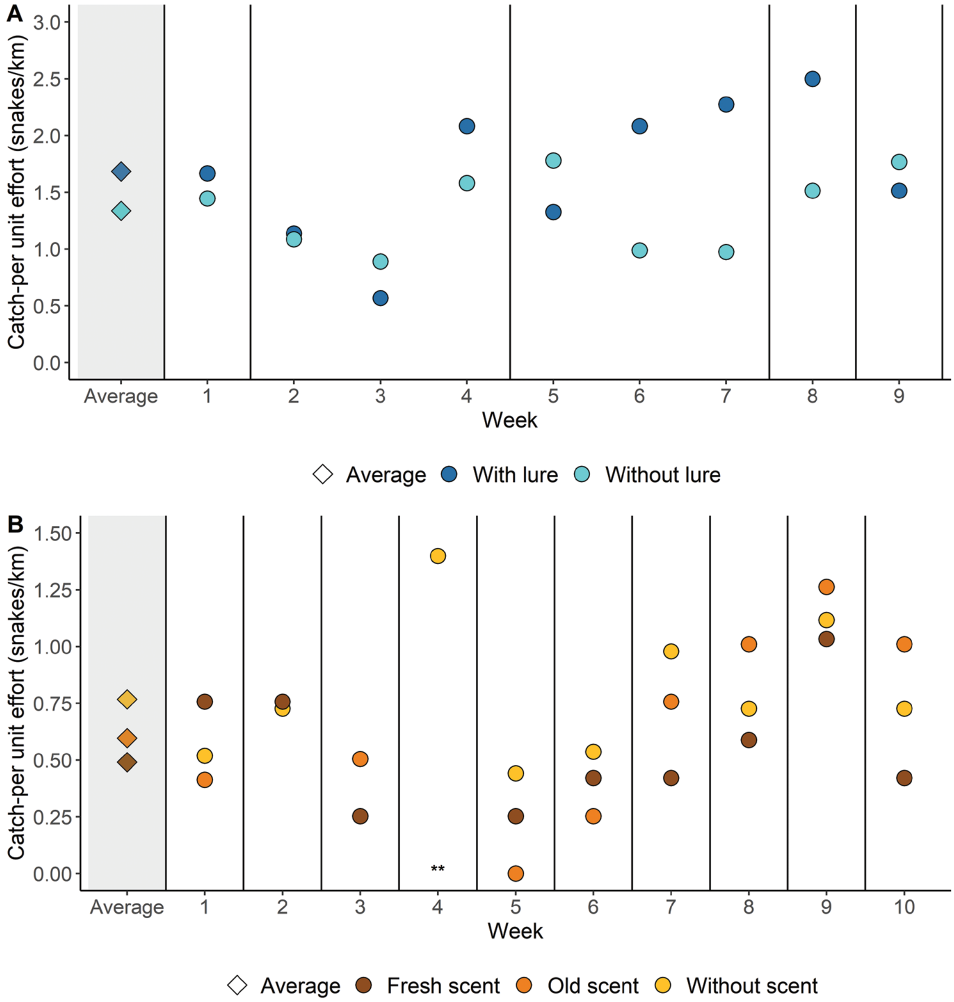

Figure 1. Weekly catch per unit effort (CPUE) of brown treesnakes along transects **A** without and with traps with mouse lures and **B** without scent, with fresh scent (applied that day), or with old scent (applied the day before). **A** Overall, average CPUE of snakes was 32% higher along transects with mouse lures than transects without such lures. Vertical lines group traps deployed at similar locations (Week 1 and 8, Week 2–4 and 9, and Weeks 5–7 denote same trap locations). **B** Overall, average CPUE of snakes was 45% and 12% higher along transects that were not sprayed as compared to those with fresh scent and old scent respectively. Asterisks indicate that no spraying occurred in week 4.

Analysis

We calculated weekly catch per unit effort (CPUE) for each project, measured as the total number of snakes caught divided by the total transect distance (km) walked during surveys each week. This metric is a commonly reported way of capturing the benefit to cost (in time) ratio of an action. We calculated this at the temporal scale of a week to better match the time frame that treatments were implemented on a transect before being rotated to a new location and to also summarize if there were any accumulated benefits to using these treatments. However, CPUE does not lend itself to statistical testing of differences, requiring further analysis of capture data.

We also analyzed the individual capture data using a spatially explicit capture mark-recapture (SCR or SECR) model (Royle et al. 2014) in a Bayesian framework. This model allows for the estimation of population abundance and density within the state space (*S*; here, the dimensions of the CP) by relating encounters of marked individuals to specific spatial locations across time, e.g., marked individual *i* at each grid cell *j* and occasion *k* (y_{ijk}). While we were not focused on estimating abundance or density, this model allowed us to estimate the detection probability for snakes on the landscape. The process model assumes there is some activity center (*s*) around which each animal in the study area uses space. As an animal moves around the landscape, its probability of being detected at a grid cell is a function of the distance between its activity center *s* and grid cell *j* and two parameters describing the encounter rate. The first parameter describes the decline in encounter probability as the distance between the grid cell and its activity center increases (σ), and the second is a baseline encounter rate at a distance of zero (i.e., λ_0, the probability that an individual lives in the grid cell in which it was caught that night).

We ran one model for each project where we allowed the baseline encounter rate ($\lambda_{STATUS_{jk}}$) to vary by the lure or scent of each grid cell *j* at the time *k*. For both analyses, we assumed a half-normal detection function such that

$$\lambda_{ijk} = z_i \lambda_{STATUS_{jk}} e^{\left(\frac{-\|s_i - x_j\|^2}{2\sigma^2}\right)} \tag{1}$$

where $||s_i - x_j||$ is the squared Euclidean distance between each activity center (s_i) and grid cell (x_j). For the first analysis, the status of a grid cell could take three forms: 1) inactive (i.e., not surveyed that evening), 2) active and without a lure (λ_{nolure}), or 3) active and with a lure (λ_{lure}). For the second analysis, the status of a grid cell could take four forms: 1) inactive, 2) active and without scent ($\lambda_{noscent}$), 3) active and with fresh scent ($\lambda_{freshscent}$), or 4) active and with old scent ($\lambda_{oldscent}$).

We used a data augmentation approach to estimate the number of individuals present in the study area but not detected during the study (Royle et al. 2014). A latent indicator variable, z_i, denotes the probability that an individual is part of the population (1) or not (0). We assumed $z_i \sim \mathit{Bernoulli}(\psi)$ for $i = 1, 2, \ldots, M$ individuals where M is a value much larger than the expected abundance. The latent indicator variable z_i limits encounters to those individuals that are part of the population, and abundance is then simply the sum of all instances where $z_i = 1$.

We fit both models using a data augmentation value of $M = 250$ and vague priors where $s_i \sim \mathit{Uniform}[S]$, $\lambda_{STATUS} \sim \mathit{Uniform}(0,1)$, $\psi \sim \mathit{Uniform}(0,1)$, and $\sigma \sim \mathit{Uniform}(0,50)$.

We ran all models using three parallel chains comprised of 1,000 adaptation iterations followed by 2,000 iterations and no burn-in or thinning. Model convergence was determined by visual inspection of traceplots and Gelman Rubin statistics ($\hat{R} \leq 1.01$; Gelman et al. 2013). Example code used to fit these models is provided in Suppl. material 1 (R code to fit the spatial capture-recapture model in JAGS).

We also calculated the probability (% of total Markov chain Monte Carlo iterations) per project that the encounter probability when using each attractant was greater or less than the encounter probability without the use of that attractant. We also calculated the mean difference between the encounter probabilities (e.g., $\lambda_{lure} - \lambda_{nolure}$).

Prediction

Using values estimated from the data that impact the way searchers detect snakes (λ_{STATUS} and σ), we simulated data to better understand the way each attractant could impact the probability of detecting snakes on a given night. For a single snake with an activity center *s* in the very center of the study area, we simulated a single evening survey where the entire study area (the same dimensions of CP; 50,000 m^2) was uniformly subjected to each of the different attractants or not (e.g., every grid cell contained a lure or not). We estimated the encounter probability at each grid cell in the study area and calculated the probability that the individual would be detected at least once in the study area when using that attractant (or lack thereof). We fit all models in JAGS (Plummer 2003) via the jagsUI package (Kellner 2018) in R (R Core Team 2019). Simulation code is provided in Suppl. material 2.

Results

Live mouse lure

During this study, we captured 100 unique individuals, with snakes being caught an average of 1.9 times (range: 1–5 times) and 3–14 snakes being caught every evening. The mean snout-vent length (SVL) of captured snakes was 918.91 mm (min = 566, max = 1205). Weekly CPUE was often higher on transects with live-mouse lures present (Fig. 1A; 0.56–2.50 snakes/km with lures as compared to 0.89–1.78 snakes/km without lures), with CPUE 32% higher on average on transects with lures as compared to those without.

Encounter probabilities of snakes in grid cells with lures was generally higher ($\lambda_{lure} = 4.26e^{-3}$ [95% credible interval {CI} = $2.98e^{-3}$, $5.82e^{-3}$]) than those in grid cells without lures ($\lambda_{nolure} = 3.25e^{-}3$ [$2.33e^{-3}$, $4.37e^{-3}$]), though 95% CIs overlapped (Fig. 2A). However, there was a 97% probability that $\lambda_{lure} > \lambda_{nolure}$, and the difference between λ_{lure} and λ_{nolure} was 0.001 ($-1.11e^{-4}$, $2.23e^{-3}$). We estimated the scale parameter, σ, to be 32.29 m (28.57, 36.53). We estimated a mean abundance of 134.31 (118, 154) snakes and a density of 27 (24, 31) snakes per ha.

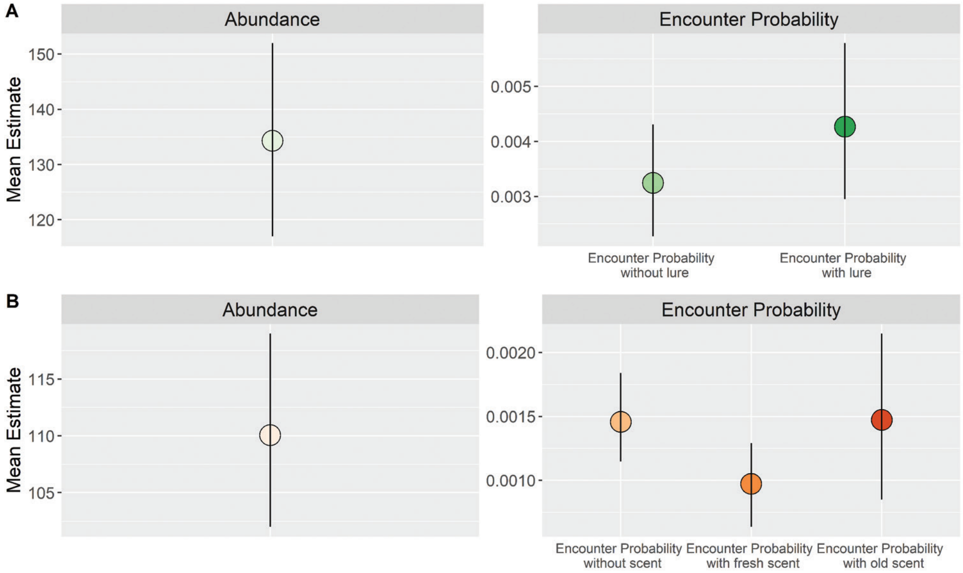

Figure 2. Mean estimated abundance (left panels) and encounter probabilities for visual surveys (right panels) on transects **A** without and with lures and **B** with different scent treatments. Lines around point estimates represent 95% credible intervals. Note that y axes are different.

Sprayed scent

In this study, we captured 96 unique individuals, with snakes being caught an average of 2.5 times (range: 1–8 times) and 2–18 snakes being caught every evening. The mean SVL of captured snakes was 950.21 mm (min = 462, max = 1203.75). Weekly CPUE was highest on transects without any scent sprayed (Fig. 1B; 0.44–1.40 snakes/km without scent as compared to 0.25–1.03 snakes/km with scent), with CPUE 45% higher than on transects with fresh scent and 12% higher than on those with old scent.

Encounter probabilities of snakes on transects that were unsprayed ($\lambda_{noscent} = 1.46e^{-3}$ [$1.14e^{-3}$, $1.83e^{-3}$]) or sprayed the day before ($\lambda_{oldscent} = 1.47e^{-3}$ [$1.14e^{-3}$, $1.83e^{-3}$]) were higher than for snakes on transects with fresh scent ($\lambda_{freshscent} = 0.97e^{-3}$ [$0.67e^{-3}$, $1.33e^{-3}$]), though again 95% CIs overlapped (Fig. 2B). During visual surveys with no scent, snakes had higher encounter probabilities than during surveys with scent, where there was a 99% and 50% probability that $\lambda_{noscent} > \lambda_{freshscent}$ and $\lambda_{noscent} > \lambda_{oldscent}$ respectively. In this case, the difference between $\lambda_{noscent}$ and $\lambda_{freshscent}$ was $4.76e^{-4}$ ($9.98e^{-5}$, $8.65e^{-4}$) and $\lambda_{noscent}$ and $\lambda_{oldscent}$ was $1.19e^{-6}$ ($-6.83e^{-4}$, $5.91e^{-4}$). Additionally, snakes on transects with older scent had a higher encounter probability than those on transects with freshly sprayed scent, with a 94% probability that $\lambda_{oldscent} > \lambda_{freshscent}$. The difference between $\lambda_{oldscent}$ and $\lambda_{freshscent}$ was $4.75e^{-4}$ ($-1.38e^{-4}$, $1.19e^{-3}$). The estimated scale parameter, σ, was slightly higher than in the lure study at 40.95 m (36.88, 45.51) and the mean estimated abundance was slightly lower at 110.07 (102, 120) snakes and a density of 22 (20, 24) snakes per ha.

Prediction

Using the estimates from the live-mouse lure component, we found that the probability of detecting a single individual on a single night (when searching every grid cell) in a study area entirely lacking a lure was 0.66 (0.55, 0.77) but increased to 0.76 (0.64, 0.86) with lures placed at every grid cell. Using estimates from the sprayed scent project, we found that the probability of detecting a single individual on a single night (when searching every grid cell) in a study area entirely lacking scent or with older scent was 0.39 (0.32, 0.47) or 0.39 (0.26, 0.53), respectively. The lowest probability of detection, 0.28 (0.21, 0.37), was in a study area with fresh scent.

Discussion

For EDRR, the probability of detecting an incipient population dictates how rapid a management response can be assessed and implemented. We tested the utility of pairing visual surveys with attractants (i.e., lures and scent) to increase the probability that searchers would encounter brown treesnakes during a rapid response effort. The CI of estimates overlapped likely due to imprecision caused by small sample sizes and limited recaptures (Fig. 2); however, our raw capture rates scaled by effort (CPUE; Fig. 1), the probabilities of having increased encounter probabilities, and the differences between encounter probabilities indicated increased snake captures on transects using live-mouse lures as attractants but not on those using spray attractants. We show that this can manifest itself in gains in the probability of detecting a snake on the landscape, with a 15% increase in the probability of detection. However, using freshly sprayed scent on transects resulted in the lowest probability of detection on a given night, decreasing the probability of detection of a snake by 28%.

When considering the efficacy of different attractants, a live-mouse lure provides both an olfactory and visual cue to brown treesnakes (Shivik 1998, though see Shivik and Clark 1997), potentially attracting snakes to, and then keeping them on, transects long enough for searchers to encounter them. On camera traps, snakes stayed in the field of view with live mouse lures for an average of 11 minutes but up to an hour in many cases and often appeared to leave and return to the lure multiple times (Amburgey et al. 2021). As such, even though a pilot study showed brown treesnakes were interested in the fish fertilizer scent, the lack of a prey item to retain attention (Lindberg et al. 2000) may result in the benefits of this attractant being highly ephemeral. Work on brown treesnakes has also shown that lipids are one of the primary components of scent attraction (Kimball et al. 2016), and fish fertilizer likely has less lipids in it than other possible attractants. Additionally, visual detection of brown treesnakes is challenging in the forests of Guåhan. For example, Savidge et al. 2011 explains how imperfect detection by human observers, even when partnered with canine detector dogs, can lead to a low number of snake captures. With scent spread out evenly across a transect and on multiple transects, searchers may not have a concentrated hotspot (i.e., a trap) upon which to focus their search or may actually deter predators with an unrewarding and confusing signal (Norbury et al. 2021), limiting the utility of this attractant.

Our estimated abundances and densities for both projects are consistent with other studies on this population (Tyrrell et al. 2009, Christy et al. 2010, Amburgey et al. 2021) in addition to the densities of snakes in forested landscapes of Guåhan (Rodda et al. 1999), indicating that encounter rates in these studies would be comparable to those in established populations of brown treesnakes. This highlights the challenge of EDRR in the case of an incipient population of brown treesnakes, as individual encounter probabilities were low ($\lambda < 5.0e^{-3}$) and effort was substantial to capture a minimal number of animals even at this high population density (Fig. 1). In our predictive simulation, substantial effort (i.e., every grid cell being surveyed in a single night) was required to achieve higher detection probabilities. Population density was slightly lower during the scent experiment and sampling occurred during the wet season, potentially explaining the overall lower encounter probabilities as compared to the lure experiment. It is also important to note that, in this study, model estimates represent survey-specific encounter probabilities while CPUE was summed over each week, potentially showing that the benefits of using attractants during EDRR may take time (or space, as shown by our predictive simulation) to accrue. For a given survey, using a lure may not drastically alter the probability of encountering a snake on a given evening; however, over time or over space, the benefits of using a lure may accumulate. Marginally higher encounter probabilities on transects with lures may potentially manifest as a benefit when multiple surveys are conducted. With such low encounter probabilities, any improvement would be beneficial. Additionally, the fact that searchers rotated lures means that there may have been some delay in the response of snakes as they must be attracted from the surrounding landscape, and there should be additional investigation of whether it is beneficial to establish permanent locations (as a reliable attractant) or rotate locations (in order to intersect more potential areas of snake use). Fed snakes also remain inactive for several days post meal consumption (Siers et al., 2018), meaning some proportion of the population will not be available for detection on a given evening and surveys should be done for long enough to ensure there are sufficient detection opportunities.

In a novel environment with high prey densities, a snake's activity status would more often be in a "fed" vs. "foraging" state and the efficacy of a lure could be limited due to an abundance of alternate prey options (Gragg et al., 2007). Snake-free islands in this region have higher prey densities as compared to Guåhan (Wiewel et al., 2009, Campbell III et al., 2012), stressing the need for further investigation of this tool in experimental situations (e.g., laboratory or simulation studies) better representing newly invaded habitats. Snake movement is also influenced by the availability of prey and density of conspecifics (Christy et al. 2017), potentially changing the area that needs to be searched around a credible sighting or capture in the case of an incipient population. This can be challenging in environments with dense forest and lack of standardized survey locations, potentially requiring the establishment of survey transects on these landscapes. Additionally, certain smaller-sized snakes may be refractory to detection and removal (Vice and Vice 2004) thus increasing the probability of them being transported to other islands. These smaller snakes are less interested in mouse lures due to size-specific shifts in predation during the brown treesnake life cycle (Lardner et al. 2009), potentially limiting the utility of these lures. However, in the case of an invasive species, such as the brown treesnake, which has caused ecological collapse and resulted in massive economic repercussions through reduced tourism, infrastructure damage, and continued interdiction and suppression efforts (Rodda and Savidge 2007), any tools that can potentially help maximize the detection of an incipient population or boost capture rates during a rapid response may be worth implementing.

Conclusion

Previous work in EDRR has highlighted the use of supplemental data types and attractants as a means to ensure detection of incipient populations that can cause massive, ecosystem-wide damage (Dejean et al. 2012; Flaherty et al. 2018; Larson et al. 2020 and resources therein). Use of attractants in a rapid response would require an explicit discussion of the direct management benefit obtained with respect to the potential costs (including obstacles of availability and quarantine procedures in the case of live animals) of deploying that method. Mouse lures may be costlier to place and maintain than a scented spray but appear to be more effective at increasing detection of snakes along surveyed transects. While the mean encounter probability on transects with mouse lures was still small, as snake traps with mouse lures are already in use at ports and airports in the region, it may be that pairing visual searches with mouse lures (inside of traps or in a different format) can provide searchers an edge while performing a rapid response. Additionally, having traps on the landscape represents a continuous opportunity to capture snakes, an additional benefit to weigh when selecting strategies to deploy. It is necessary for managers to explicitly consider the costs of deploying and maintaining traps and transects with regards to the potential gains when selecting a strategy.

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or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. All code and data to run these analyses are available at https://github.com/amburgey/Browntreesnake_ATTRACTANTS.

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Supplementary material 1

R code to fit the spatial capture-recapture model in JAGS

Authors: S.M. Amburgey, A.A. Yackel Adams, B. Gardner, B. Lardner, A.J. Knox, S.J. Converse

Data type: model code

Explanation note: R code to fit the spatial capture-recapture model in JAGS. Code example is for the mouse lure project but was similar to that used for the spray scent project. Simulated data are included for reference as to the structure and form of data input into the model.

Link: https://doi.org/10.3897/neobiota.70.71379.suppl1

Supplementary material 2

Code used to simulate detection probabilities

Authors: S.M. Amburgey, A.A. Yackel Adams, B. Gardner, B. Lardner, A.J. Knox, S.J. Converse

Data type: model code

Explanation note: Code used to simulate detection probabilities and observations of a single snake in the study area based on estimated parameters (from JAGS model, saved as "out"). Example code shows calculations for mouse lure predictions but is similar to that used for spray scent predictions. By using all the samples in the posterior, we estimated uncertainty.

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RESEARCH ARTICLE

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Management of invasive alien species in Spain: A bibliometric review

Rafael Muñoz-Mas[1], Martina Carrete[2], Pilar Castro-Díez[3], Miguel Delibes-Mateos[4], Josep A. Jaques[5], Marta López-Darias[6], Manuel Nogales[6], Joan Pino[7,8], Anna Traveset[9], Xavier Turon[10], Montserrat Vilà[11,12], Emili García-Berthou[1]

1 *GRECO, Institute of Aquatic Ecology, University of Girona, 17003 Girona, Spain* **2** *Departamento de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, Ctra. de Utrera, km. 1 41013, Seville, Spain* **3** *Biological Invasions Research Group (BioInv), Departamento de Ciencias de la Vida, Universidad de Alcalá, Pza. San Diego, s/n, 28801, Alcalá de Henares, Madrid, Spain* **4** *Instituto de Estudios Sociales Avanzados (IESA-CSIC), Plaza Campo Santo de los Mártires, 7, 14004 Córdoba, Spain* **5** *Departament de Ciències Agràries i del Medi Natural, Universitat Jaume I, Av. Vicent Sos Baynat, s/n, 12071 Castelló de la Plana, Spain* **6** *Island Ecology and Evolution Research Group, Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Avda. Astrofísico Francisco Sánchez, 3, 38206 - San Cristóbal de La Laguna Santa Cruz de Tenerife – Islas Canarias, Spain* **7** *CREAF, E08193 Bellaterra (Cerdanyola del Vallès), Catalonia, Spain* **8** *Universitat Autònoma de Barcelona, E08193 Bellaterra (Cerdanyola del Vallès), Catalonia, Spain* **9** *Instituto Mediterráneo de Estudios Avanzados (IMEDEA,CSIC-UIB), C/ Miquel Marquès, 21 - 07190 Esporles , Mallorca, Illes Balears, Spain* **10** *Departamento de Ecología Marina, Centro de Estudios Avanzados de Blanes (CEAB-CSIC), Accés a la Cala St. Francesc, 14, 17300 Blanes, Spain* **11** *Estación Biológica de Doñana (EBD-CSIC), Avda. Américo Vespucio 26 ,41092 Sevilla, Spain* **12** *Department of Plant Biology and Ecology, University of Sevilla, 41012 Sevilla, Spain*

Corresponding author: Rafael Muñoz-Mas (rafa.m.mas@gmail.com)

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Abstract

Scientific and grey literature on invasive alien species (IAS) is conditioned by social, economic and political priorities, editorial preferences and species and ecosystem characteristics. This leads to knowledge gaps and mismatches between scientific research interests and management needs. We reviewed the literature on IAS management in Spain found in Scopus, Web of Science, Google Scholar and Dialnet to identify key deficiencies and priority research areas. The collected literature was classified, employing features describing formal aspects and content. We used bibliometric and keyword co-occurrence network analyses to assess the relationship between features and reveal the existence of additional topics. Most of the compiled documents (n = 388) were focused on terrestrial ecosystems and inland waters, whereas marine and urban ecosystems were under-represented. The literature was largely generic and not species-specific, focusing on raising awareness and proposing changes on current regulation as prominent approaches to prevent further introductions. The compiled authors exhibited many clear publishing preferences (e.g. language or document type), but less regarding target taxa. In addition, there was a strong association between species and the different features considered, especially between the methodological approach (e.g. review, field experiment) and the primary emphasis of study (i.e. basic/theoretical, applied or interdisciplinary). This indicates that research on IAS has had a strong species-specific focus. References about terrestrial species focused mainly on vascular plants, whereas references about inland waters were mostly on fishes and the giant reed (*Arundo donax*), which has been managed with partial success. Animal culling and plant removal were the most frequent eradication and small-scale control treatments, whereas the documents addressing wider spatial scales were largely theoretical. Consequently, the success of described treatments was largely uncertain. Spanish invasion science research has been occasionally innovative, incorporating novel technologies (e.g. species distribution modelling) and engaging society with citizen-science approaches. However, the ratio between basic/theoretical and applied studies indicates that more applied research/management is needed, especially in inland waters and marine ecosystems. We call for increasing effort in the effective dissemination of experience in IAS management to enhance current practical knowledge, including that of schemes undertaken by public agencies.

Keywords
Biological invasions, eradication, control, Iberian Peninsula, Mediterranean, Spanish archipelagos, keyword co-occurrence analysis, prevention

Introduction

Biological invasions are human-assisted global phenomena with ravaging effects, not only on biodiversity and ecosystem services, but also on human well-being (McGeoch et al. 2010; Vilà and Hulme 2017). Although transport and introduction of alien species into novel ecosystems is inherent to humankind's expansion (Crees and Turvey 2015), the number of new introductions has increased exponentially since the mid-twentieth century (Seebens et al. 2018, 2019). Invasive alien species (IAS) can reshape ecosystem processes, decrease native species richness and abundance (e.g. McGeoch et al. 2010 and references therein) and cause impact on the economy and human health (Zenni et al. 2021). Thus, preventative, eradication and control actions are required to impede their entry and establishment or minimise their long-term impacts (Robertson et al. 2020).

The incidence of biological invasions and their related costs have led to substantial management efforts worldwide to prevent new introductions and control those already established, by means of eradication or containment (García-de-Lomas and Vilà 2015; Diagne et al. 2021). However, to offer efficient responses is challenging and, regrettably, the specific literature on IAS management is often neglected during the decision-making process (Walsh et al. 2015). In Spain, managers, consultants and assessors face obstacles to find and access suitable references, as a large proportion of papers are written in English, are too scientifically orientated or narrowly focused to be directly applied or are not open-access (Andreu and Vilà 2007; Mungi et al. 2019; Copp et al. 2021). In addition, many applied studies are scattered amongst the grey literature (often in many different languages), tending to be largely inaccessible to international readers, which further limits the transfer of knowledge on both local and international levels (Haddaway and Bayliss 2015, Jeschke et al. 2019).

In addition to accessibility barriers, the content of scientific literature is also taxonomically and geographically biased (Hulme et al. 2013). This is accentuated by the fact that reviews and positive rather than negative results are more likely to be published (Fanelli 2012). Thus, several characteristics inherent to IAS and recipient ecosystems utterly favour the availability of literature on specific taxa, regions or ecosystems that are easier to study and/or manage (Thomsen et al. 2014; Nghiem et al. 2016; Shackleton et al. 2019). Moreover, trends in invasion science are also affected by social and political priorities, which are, in turn, influenced by communication media and the outcomes of previous research (Gläser and Laudel 2016; Geraldi et al. 2019; Shackleton et al. 2019). This can lead to changes in the importance of research topics over time, for instance through fostering a given species over others or by abandoning certain research areas.

Bibliometric and keyword co-occurrence network analyses are useful to reveal the main knowledge components of any discipline, such as areas with deep insights, outstanding gaps and peripheral research areas (Aria and Cuccurullo 2017, Radhakrishnan et al. 2017). Despite some recent contributions (Enders et al. 2019, 2020), such analyses are scarce in previous reviews of IAS literature, particularly related to management. Consequently, we chose to use them to identify the main patterns in the scientific literature shown by IAS management in Spain. Our specific aims were to: (i) characterise the species, topics and approaches from a management viewpoint, (ii) detect relationships between them and (iii) identify research areas deserving further attention. We focused on Spain due to its diversity of climates and ecosystems, along with its insular and continental territories, which have favoured the establishment of a large and diverse number of IAS (Muñoz-Mas and García-Berthou 2020). Mainland Spain is part of the Mediterranean biodiversity hotspot (Williams et al. 2011) and encompasses an enormous range of ecosystem types, from arid coastal regions to mountain ranges and woodlands. Spain includes two markedly-different populated archipelagos: the Mediterranean Balearic Islands and the Macaronesian Canary Islands off the north-western African coast (Andreu et al. 2009; Benito-Calvo et al. 2009). Islands are in themselves biodiversity hotspots, but they have especially suffered from the establishment of IAS, which have caused numerous extinctions (Lenzner et al. 2020). In addition, Spain has two autonomous cities located on the Mediterranean coast of the African continent, which may require different IAS management approaches. Former reviews on invasion management in the country were sectorial and focused on stakeholder perceptions and management of alien plants (Andreu et al. 2009) or were based on the most common deficiencies in IAS management (Dana et al. 2019). Thus, reviewing past and present experience may help to improve current IAS management actions and identify future research lines in Spain and similar territories. Our results may help funding agencies to target as yet unidentified research needs.

Methods

Literature review

We performed our literature search using Scopus, Web of Science (WoS) and Google Scholar. The first two mainly focus on English language literature, largely scientific papers, whereas the references compiled within Google Scholar are more heterogeneous and less structured (Haddaway et al. 2015). Monolingual searches have been shown to limit and bias results in global literature reviews (Angulo et al. 2021; Nuñez and Amano 2021). To avoid such potential bias, in addition to Google Scholar, we also consulted Dialnet. This is a multidisciplinary reference database launched in 2001 that focuses on scientific literature published in Spanish, including books, theses and other documents.

We used the following search terms in Scopus and WoS (both accessed 28 April 2020): (Spain OR Iberian Peninsula OR Canary Islands OR Balearic Islands OR Ceuta OR Melilla) AND (alien species OR exotic species OR non-native species OR invasive species OR introduced species OR species introduction OR translocated species OR species translocation OR species spread OR naturalised species OR casual species OR species of concern OR noxious species OR pernicious species OR harmful species) (Rytwinski et al. 2020). We also used the equivalent Spanish terms for searches in Google Scholar and Dialnet, the latter accessed 30 May 2020. The results were downloaded from Scopus, WoS and Dialnet and duplicates removed (Rytwinski et al. 2020) (Table 1). Following Rytwinski et al. (2020), we modified this general workflow for searches in Google Scholar to deal with the inferior specificity of the searching engine. Between 29 April and 30 May 2020, we retrieved up to 1,000 documents, including duplicates, starting the search using the Spanish keywords describing the toponymy (e.g. España or peninsula Ibérica) and terms used to name IAS (e.g. especie exotica or especie invasora) with one of the following terms: gestión (management), erradicación (eradication) or control. Starting with gestión, we selected documents up to the point when they were clearly irrelevant or duplicated. We then repeated the query using the following keyword (first erradicación and finally control) and repeated the entire process using the English keywords. Once 1,000 documents were compiled, the full text was checked to discard irrelevant documents and, only then, we added the document to the references obtained from Scopus and WoS (Table 1).

Only documents focusing on direct IAS management (i.e. eradication and control) or with explicit management implications in Spain were included (e.g. risk assessment and prevention of future invasions, regulations or education). Studies exclusively focusing on the biology or the ecology of IAS, with no management implications or with no examples, were discarded. We only counted introduced and established species or species able to spread in the wild (e.g. Blackburn et al. 2011), excluding those in captivity or cultivated. We also considered microorganisms and parasites (potentially) affecting other species in the wild (e.g. the crayfish plague *Aphanomyces astaci* or the amphibian chytrid fungus *Batrachochytrium dendrobatidis*), but excluding agricultural pests or pathogens of captive animals or humans.

In general, no agriculture-related studies were retained, nor weeds interfering with crops or plantations. However, studies on the red weevil, *Rhynchophorus ferrugineus*, were not excluded, although they focused on the date palm *Phoenix dactylifera*, because this weevil also affects the endemic Canary Islands date palm *Phoenix canariensis*. We compiled a total of 388 documents (Table 1).

Literature characterisation and keyword compilation

We selected relevant features of the retrieved documents for our analysis, following previous bibliographic studies on IAS management (mostly Bayliss et al. 2013; Matzek et al. 2014) (Table 2). The selected features described formal aspects (e.g.

Table 1. Total number of references retrieved before scrutiny (e.g. including duplicates) and final number of references retained for the database. Web of Science and Scopus were managed simultaneously because they show a great overlap of hosted references.

Bibliographic database	Number of references retrieved	Number of references retained
Scopus	1569	214
Web of Science	1152	
Dialnet	75	62
Google Scholar	1000	112

Table 2. Features and categories used to characterise the compiled literature (based on Andreu and Vilà 2007; Bayliss et al. 2013; Matzek et al. 2014). Specific definitions for each category of the selected features are provided in Supplementary Material (Table S1).

Feature	Categories
Authors	–
Document type	Scientific article, book, book chapter, conference proceedings, report or thesis
Ecological organisation level	Population/autoecology, community/assemblage, ecosystem or generic/not applicable
Ecosystem type	Terrestrial, inland waters, marine or urban
Insular territory	Yes or no
Language	English or Spanish
Main topic	Management (eradication and control), prediction, prevention, prioritisation (including risk management), regulation/decision-making, risk assessment, social, biology/ecology, climate change and other synergisms, conservation, definitions, economics, education and awareness-raising, impacts, information sharing, communication and collaboration, introduction/spread or survey/monitoring
Management topic	Prevention, eradication, control or generic/not applicable
Methodological approach	Review/meta-analysis, observational field study, field experiment, greenhouse/laboratory experiment, opinion paper or theory
Primary emphasis	Basic (without direct manipulation of the target taxa), applied (with direct manipulation of the target taxa) or interdisciplinary (encompassing social and policy issues)
Protected area	Yes or no
Spatial scale	Local, regional, national, supranational/global or other/undefined (e.g. greenhouse/lab experiment)
Species	Target species, taxon or generic/multiple taxa
Success	Yes, partial, no, unknown or not applicable
Treatment	Regulation, awareness raising, culling, biocontrol agent, poisoning, hydrology manipulation, hydrogeomorphological restoration, survey and monitoring, mechanical removal, manual removal, herbicide, genetic selection, exclosure, not applicable, DNA metabarcoding, re-afforestation, heating, baiting, containment, mulching, prevention, restocking, incineration, insecticide or sterilisation
Year	-

document type or text language) and content (e.g. methodological approach, main topic, spatial scale). To better characterise documents with multiple topics and sections, the categories within the different features were non-exclusive. That is, a document could focus on the biology/ecology, introduction/spread and survey/monitoring of IAS or on prevention, eradication and control. However, during subsequent analyses, we downweighed each reference in the frequency-related calculations to sum up to one and ensure the equal contribution of all documents (Muñoz-Mas and García-Berthou 2020).

We downloaded the keywords from documents retrieved from Scopus and WoS and manually scrutinised those documents compiled from Google Scholar and Dialnet to compile the available keywords. Then, we inspected the keywords to detect mistakes and misspellings and translated those words into Spanish using Google Translator, adjusting English keywords to well-established terms when necessary (e.g. caña común/common reed to giant reed). The references and features can be found in Supplementary Material.

Data analyses

We used the cumulative sum of number of documents per year to compare the publishing trends in management of invasive alien species in Spain with more general science publishing trends (in Spain and worldwide). The total number of documents published worldwide and in Spain were those included in the Journal Citation Reports (JCRs). The series were retrieved from: www.scimagojr.com/countryrank.php. Frequency bar plots enabled scrutiny of the prevalence of the different categories of each additional feature, except species and ecosystem types. We investigated these two features simultaneously using the function comparison.cloud of the R (R Core Team 2021) package wordcloud (Fellows 2018), but without graphical scrutiny for language, insular territory or protected area, due to their binary nature.

The association amongst features was analysed, except year (Table 2), employing Cramér's V Index (Cramér 1946), implemented in the R package oii (Hale et al. 2017). This Index ranges from 0 (no association) to 1 (perfect association) and is based on a corrected χ^2 statistic. To graphically describe the association patterns, we built a network using the R package igraph (Csardi and Nepusz 2006), employing the values of the Index only when statistically significant ($P < 0.05$).

Cramér's V Index evaluates the association between features, but provides no information about the relationship amongst categories. Therefore, we built two alluvial diagrams to graphically scrutinise the relationship between the categories of the features: methodological approach, ecosystem type, management topic and spatial scale and ecosystem type, treatment and success (Table 2). Alluvial diagrams are a kind of Sankey diagram that group together observations of the same category and visualise them as flows across the considered set of features (Rosvall and Bergstrom 2010). We used the function SankeyDiagram in the R package flipPlots (Displayr 2019) to build the alluvial diagram.

To investigate the existence of additional topics and research areas not described by the features and categories detailed in Table 2, we analysed the literature using a keyword co-occurrence network (Radhakrishnan et al. 2017). As customary, we first systematically lemmatised/stemmed the resulting keywords in R (i.e. inflected or derived words were reduced to their root form) to reduce the variability within the collected keywords. For example, by applying this procedure, the word biolog would result from the words biological and biology. For this, we used the function word-Stem of the R package SnowballC (Bouchet-Valat 2020) to allow reproducible results. Compound keywords were split (e.g. invasive species was divided into invasive and species), each word was lemmatised independently (e.g. invas and speci) and the resulting words were reassembled (e.g. invas speci) to build the co-occurrence network. The co-occurrence matrix cross-product was obtained via the function cocMatrix of the R package bibliometrix (Aria and Cuccurullo 2017) and we built the co-occurrence network with igraph (Csardi and Nepusz 2006). We delineated the relevant research areas or clusters employing the function cluster_edge_betweenness (Newman and Girvan 2004) in igraph (Csardi and Nepusz 2006). Finally, the most frequent keyword of each cluster was used to simplify the complete network into a smaller network and facilitate its interpretation. To avoid oversimplification of the network, we kept the most frequent keywords (> Q_{95} or number of occurrences ≥ 4 occurrences), while the less frequent keywords were collapsed to the most frequent keyword in the corresponding research area (i.e. the cluster centre). We depicted the resulting network with the most important keywords in each research area (i.e. cluster centres encompassing the most frequent and less frequent keywords into single vertices and additionally those keywords whose frequency of occurrence was ≥ 4) as two-level circular treemaps (Zhao and Lu 2015) using the R package ggraph (Pedersen 2021).

Results

The number of documents published on IAS management has grown steadily since 1995 (Figure 1A). However, those published in Spain are under-represented compared to the scientific production trends both globally and in Spain, although during the last decade, the scientific production accelerated significantly. Most documents were scientific articles (72.4%) (Figure 1B). Review/meta-analysis was the most common methodological approach (54.9% out of the 388 documents), followed by field experiment (19.0%) and observational field study (18.9%) (Figure C). The total number of authors was 1,280. The most prolific author was Montserrat Vilà (13 documents/3.4%), followed by Pilar Castro-Díez, Elías D Dana and Juan García de Lomas (7 documents each/1.8%), whereas 1,067 authors appeared in one single document (Figure 1D). Most documents were written in English (59.3%) and the remainder in Spanish.

Management (i.e. eradication and control) was the most frequent topic (31.8% out of 388 documents), followed by risk assessment (9.6%), survey/monitoring (9.3%), biology/ecology (7.6%), and impacts (7.4%) (Figure 2A). Studies focusing on populations and the autoecology of a single species were the most frequent (45.2%), followed by studies at the community/assemblage level (24.0%) (Figure 2B). The dominant

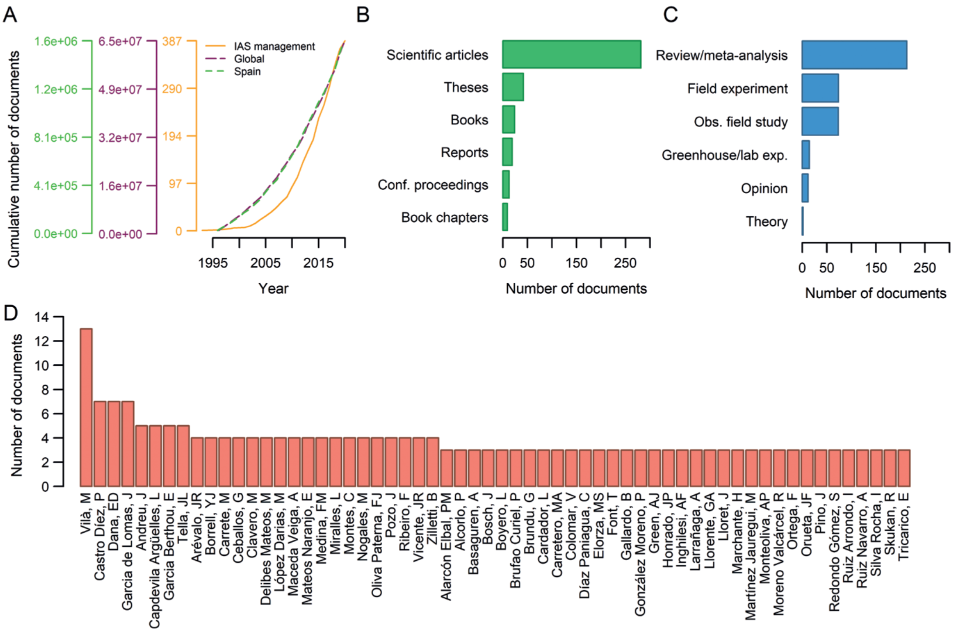

Figure 1. Cumulative number of documents on IAS management in Spain and cumulative number of scientific documents included in the Journal Citation Reports (JCRs), with no distinction by research area (**A**). Global series encompasses all the documents included in the JCRs, whereas the Spain series includes exclusively those produced in Spain (data retrieved from www.scimagojr.com/countryrank.php). Numbers of documents by types are represented in (**B**), methodological approaches in (**C**) and number of documents by the most prolific authors in (**D**).

management topic was generic/not applicable (48.0%) — i.e. documents that did not focus on prevention, eradication or control of IAS and did not involve direct manipulation of target IAS — followed by control (22.7%), whereas studies focusing on prevention and eradication were less frequent (17.5% and 11.7%, respectively) (Figure 2C). Studies on a local or national scale were more frequent (28.9% and 24.2%) than those on the intermediate (regional) or largest (supranational/global) scales (20.6% and 18.6%, respectively) (Figure 2D). The most frequent primary emphasis was basic (i.e. without direct manipulation of target taxa) (45.6%), followed by applied or interdisciplinary scopes (i.e. encompassing social and policy issues), both with similar prevalence (28% and 25.5%, respectively) (Figure 2E). The most frequent management treatments concerned impeding the establishment of further IAS through awareness-raising, regulation and survey monitoring (18.0%, 15.3% and 11.8%, respectively) (Figure 2F). Animal culling (9.1%) and plant mechanical and manual removal (11.5%) were the most common eradication and control treatments, whereas 8.2% of documents did not report specific treatments of target IAS (i.e. not applicable). The success of the proposed treatments as eradication or control methods was largely uncertain (not applicable/42.8% or unknown/23.4%) or partial (15%), which implies

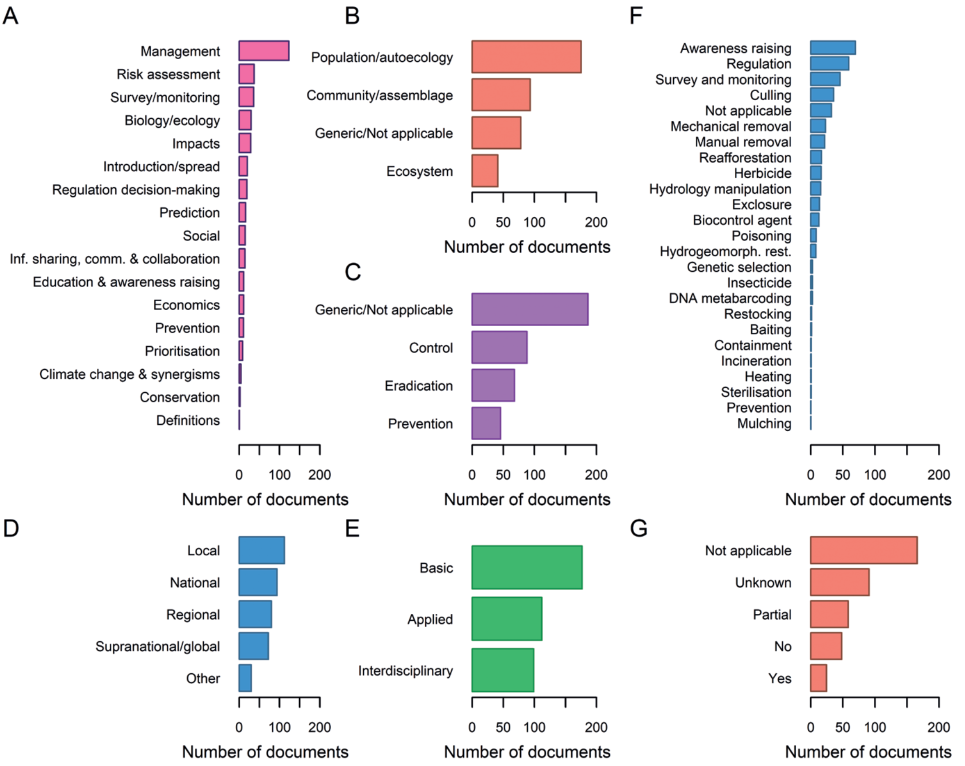

Figure 2. Numbers of documents by main topics are represented in (**A**), ecological organisation levels in (**B**), management topics in (**C**), spatial scales in (**D**), primary emphasis in (**E**), treatment in (**F**) and success in (**G**).

sustained management actions to control the target IAS (Figure 2G). About 13% of the studies focused exclusively on insular territories and 17% on protected areas.

The compiled studies involved 159 species or higher taxa (e.g. vascular plants). The largest number dealt with terrestrial ecosystems (50.4%), followed by inland waters (36.2%), whereas marine ecosystems and urban environments were the least common (8.3% and 5.0%, respectively) (Figure 3). Terrestrial vascular plants (10.4%) were the most frequent, especially eucalypts (*Eucalyptus* spp.) (1.6%), prickly pear species (*Opuntia* spp.) (1.3%), Hottentot figs (*Carpobrotus* spp.) (1.3%), Monterey pine (*Pinus radiata*) (1.3%) and cordgrasses (*Spartina* spp.) (1.0%). Amongst terrestrial animals, the most frequent species were the yellow-legged hornet (*Vespa velutina*) (1.6%), the pinewood nematode *Bursaphelenchus xylophilus* (1.3%), American mink *Neovison vison* (1.0%) and generic studies on vertebrates (3.3%), mainly birds (1.3%). In inland waters, generic studies were also the most common (7.5%), followed by those on fish management (7.0%). The most studied species in inland waters were the giant reed (*Arundo donax*) (3.1%) and the red swamp crayfish (*Procambarus* clarki) (1.8%), followed by generic studies on riparian vegetation (1.6%). Most studies on marine

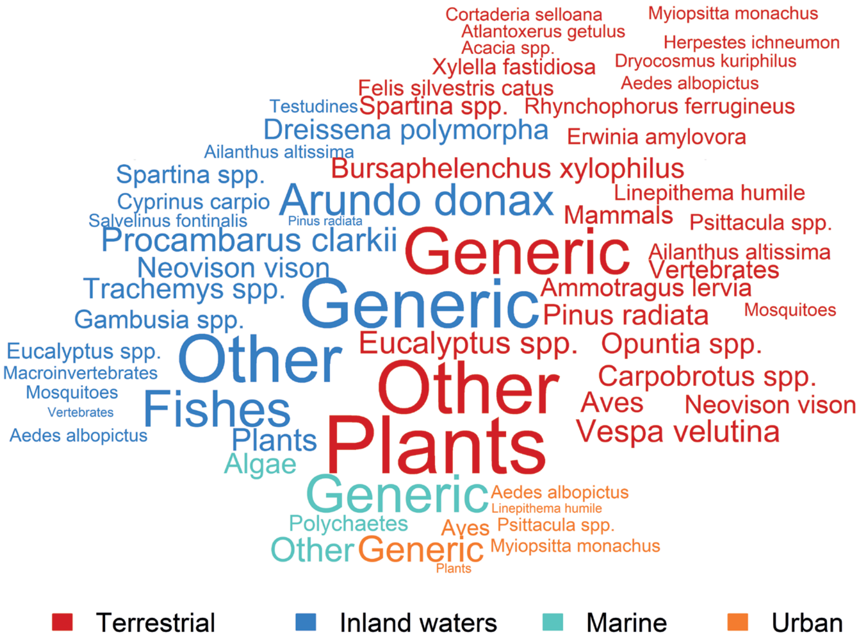

Figure 3. Word cloud depicting the proportion of documents per ecosystem type and taxon obtained using the function comparison.cloud of the R package wordcloud (Fellows 2018). The species or taxa occurring in less than two documents have been grouped within the category Other. Frequencies have been square root-transformed to facilitate reading the species.

environments were generic (4.4%) or focused on algae (1.8%) or polychaetes (1.3%). In urban environments, generic (2.1%) and bird studies (0.8%) prevailed, in addition to those on tiger mosquito (*Aedes albopictus*) (0.5%).

The association network displaying Cramér's V Index between pairs of features indicated great specificity regarding authors' preferences about publishing language and document type and on the management scales and treatment success of the conveyed experiences and approaches (Cramér's V > 0.91), but inferior regarding the target species (Cramér's V = 0.76). Subsequently, the association network highlighted the strong association between species and all other features (Cramér's V > 0.50), except with document type and protected area (Figure 4). The highest association of this group occurred between species and ecosystem type (Cramér's V = 0.66) and between species and insular territory (Cramér's V = 0.65). The connections between species, methodological approach and primary emphasis, as well as those amongst the latter two, were noteworthy. There was no strong association between the remaining features, except between the main topic and primary emphasis and the management topic (Cramér's V > 0.50). Insular territory and especially document type depicted the lowest association with the remaining features.

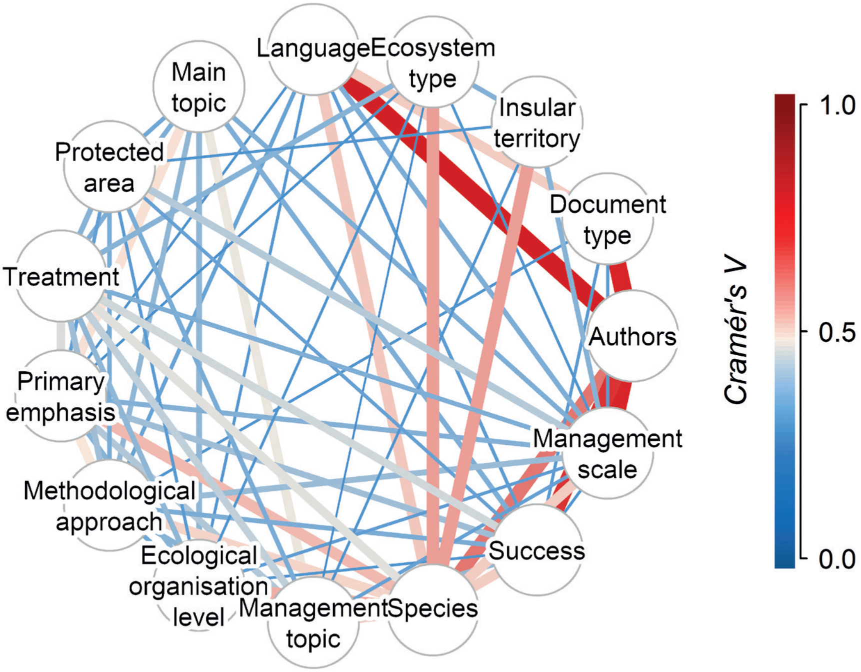

Figure 4. Association network displaying Cramér's V between features characterising the documents on IAS management in Spain. Connections are only depicted when the χ^2 statistic is significant ($P < 0.05$). Connection width and colour are based on Cramér's V and range from 0 (no association) to 1 (perfect association).

The most frequent studies were reviews and meta-analyses focusing on terrestrial ecosystems and providing general guidelines to manage invasive species at the national (6.5% out of the 388 documents), supranational/global (5.3%) and regional scales (4.3%) (Figure 5). They were followed by field experiments (manipulative) on terrestrial ecosystems addressed to locally eradicate specific taxa (3.3%). Reviews and meta-analyses on inland waters and those providing general guidelines to manage IAS at higher scales (i.e. regional to supranational/global) were also frequent (3.2% and 2.8%, respectively). Field experiments (manipulative) in inland waters addressed to eradicate (3.0%) or control (2.8%) specific taxa were in all cases local. The most frequent observational field studies on both terrestrial ecosystems (2.3%) and inland waters (2.0%) addressed generic aspects and were local. In contrast, documents focusing on marine ecosystems were scarce and studied generic aspects of marine invasion science with supranational/global (1.0%) or local (0.9%) perspectives. Manipulative field experiments to locally eradicate marine species were markedly rare (0.3%). Studies in urban environments were mostly reviews and meta-analyses addressing generic aspects at both supranational/global and local scales were the most abundant (0.5% and 0.5%, respectively). The proportion of field manipulative experiments to eradicate or control species was negligible.

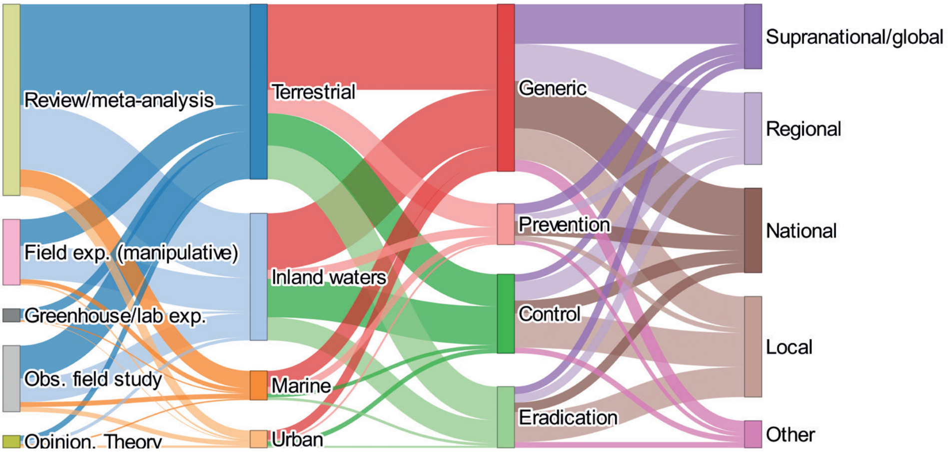

Figure 5. Alluvial diagram relating the methodological approach, ecosystem type, management topic and spatial scale of the compiled documents on invasive alien species (IAS) management in Spain. Connection width is proportional to the number of documents (An interactive version of this figure can be downloaded from https://doi.org/10.6084/m9.figshare.16547790.v1).

The most frequent approaches, described within terrestrial studies, focused on awareness-raising, survey/monitoring and regulation (7.3%, 6.8% and 3.1%, respectively out of the 388 documents). Most of them had no quantifiable (not applicable/17.2%) or unknown success (2.1%). This was followed by the use of biocontrol agents whose efficacy has not yet been tested (i.e. unknown, 1.6%) and unsuccessful culling of IAS (none and partial, 1.7%). The successful approaches most frequently reported combined herbicides and mechanical and manual plant removal (2.9% in total). In inland waters, awareness-raising and regulation were amongst the most frequently indicated approaches (3.9% and 3.0%, respectively), although with no quantifiable success (not applicable, 6.9%). Water level and flow regime manipulation was the most frequent management approach, but it was not tested (unknown, 2.3%) and occasionally turned out useless (0.7%). Culling and poisoning seldom worked (0.8% and 0.5%, respectively) and the success of most reported experiences was unknown or partial (1.6% and 0.9%, respectively). Hydrogeomorphological restoration, re-afforestation, plant removal and herbicide use were the most common approaches to control invasive riparian vegetation (1.2%, 1.1%, 1.6% and 0.7%, respectively). However, success of these treatments was partial or uncertain and only 0.6% reported successful experiences. The treatments for marine ecosystems followed a similar pattern and focused on preventative approaches: awareness-raising, survey/monitoring and regulation (1.9%, 1.0% and 0.9%, respectively), most of them with no quantifiable (not applicable/2.0%) or unknown success (1.6%). The only successful study involved raising awareness and DNA metabarcoding to confirm the elimination of the pygmy mussel (*Xenostrobus securis*) (Miralles et al. 2016). Awareness-raising, regulation and survey/monitoring were the most common approaches for urban environments (3.7% in total), but the few applied experiences indicated partial success through culling and plant removal and subsequent herbicide spraying (0.3%).

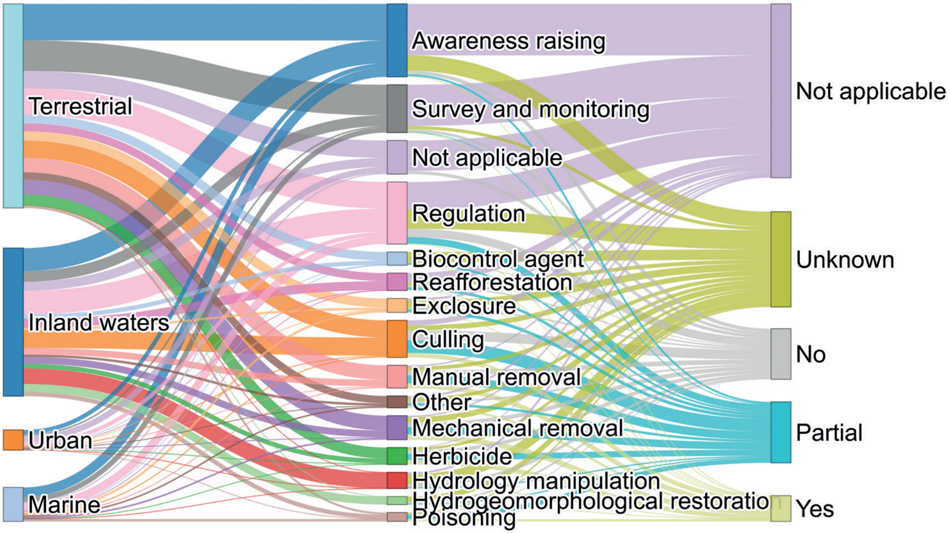

Figure 6. Alluvial diagram relating the ecosystem type, treatment and success of the compiled documents on invasive alien species (IAS) management in Spain. Connection width is proportional to the number of documents. The category Other includes mulching, prevention, sterilisation, heating, incineration, containment, baiting, restocking, DNA metabarcoding, insecticide and genetic selection (Alternative static and interactive versions of this figure relating species/taxa, treatment and success can be downloaded from https://doi.org/10.6084/m9.figshare.16547790.v1).

The collected keywords encompassed 1,145 different terms. The aggregation algorithm revealed 39 different research areas or clusters (Figure 7A), with 67 keywords occurring on ≥ 4 occasions (Figure 7B). The largest research area highlighted the importance of invasive plants amongst the Spanish literature on IAS management, the Mediterranean nature of much of the territory and the numerous studies carried out on this taxon in the Balearic Islands. The simplified network reflected the main topics of the study: invasive species and management, eradication and control. It also reflected research carried out on specific taxa, such as on the elimination of American mink (*Neovison vison*), which clustered with invasive species. Exclusion experiments on the European rabbit (*Oryctolagus cuniculus*) and feral cats (*Felis silvestris catus*) appeared in different research areas. The studies on feral cats were undertaken mainly in the Canary Islands as their research areas were connected. In addition, the resulting network highlighted the impacts on freshwater biodiversity caused by giant reed (*A. donax*) and aquaculture activities. The simplified network reflected the importance of the yellow-legged hornet (*V. velutina*) and mosquitoes (mainly the tiger mosquito *A. albopictus*) and the extensive use of species distribution models (SDMs), such as MaxEnt (Phillips et al. 2004), to foresee expansion trends and suitable regions. Specific topics (propagule pressure and ecosystem services) and introduction vectors (ballast waters, aquaculture and hunting) appeared in separate research areas. The simplified network reflected specific management approaches, such as general forest management, use of herbicides or emergence of citizen science. It also reflected studies addressing the interaction be-

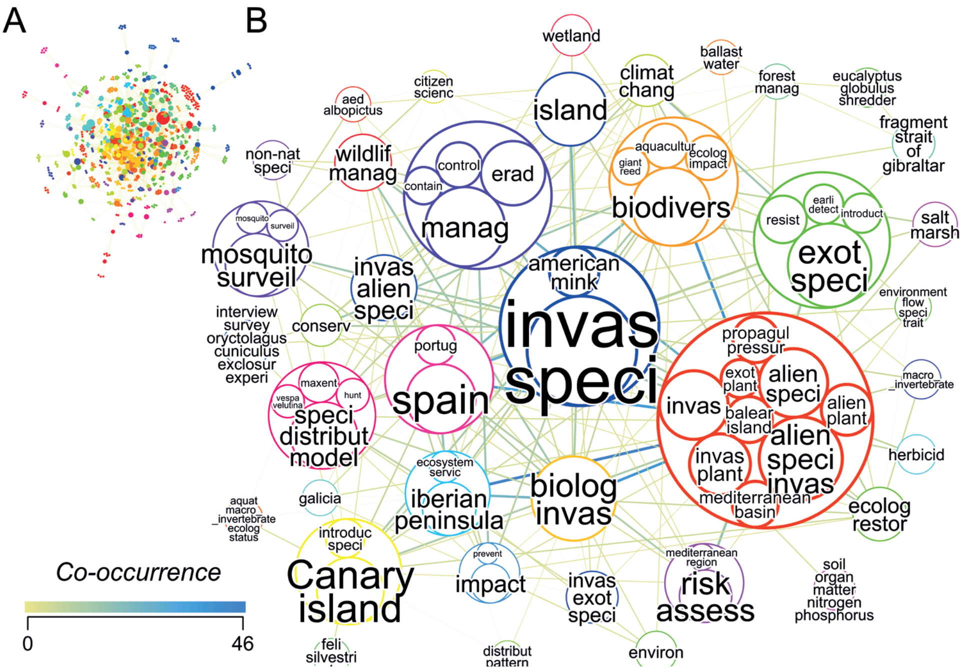

Figure 7. (**A**) Complete keyword co-occurrence network developed to visualise the importance of the research areas. Vertex colours are based on research areas or clusters and vertex size is proportional to the frequency of keyword occurrence. (**B**) Simplified network based on cluster centres and most frequent keywords (> Q_{95} or number of occurrences ≥ 4). Overall vertex sizes are proportional to the log-transformed number of occurrences and inner circles to the proportion within each research area. Label sizes have been rescaled to avoid overlapping.

tween invasions and climate change. In addition to the main toponymy used during the bibliography search, the network reflected specific regions and environments, such as the Strait of Gibraltar and Galicia (NW Spain) and the importance of wetlands and salt marshes. Moreover, the simplified network highlighted the importance of the archipelagos and islands within Spanish invasion science research, given that island and Canary Islands appear in differentiated research areas. It also revealed the multiplicity of terms used to name similar concepts, such as the terms alien species and exotic species that appear scattered throughout different research areas.

Discussion

Spanish literature on IAS management has been mainly theoretical (55% review/meta-analysis), with a balance between theoretical and applied studies similar to that reported in other studies on biological and ecological aspects of biological invasions (Andreu and Vilà 2007; Bayliss et al. 2013; Matzek et al. 2014, 2015). Nonetheless, IAS management literature has been under-represented compared to the Spanish and global trends on scientific production, although recently its share has grown. Altogether, this suggests that Spanish invasion science may also suffer from a knowing-doing gap caused by a preponderance of theoretical studies (Matzek et al. 2014, 2015). Moreover, scientific papers written in English constituted a large proportion of the compiled literature, which suggests that most of it may be too scientifically orientated to be directly applied (Andreu and Vilà 2007; Mungi et al. 2019; Copp et al. 2021). Nevertheless, scientific activity in Spain has also incorporated new forecasting technologies (e.g. SDMs/MaxEnt, de Medeiros et al. 2018) along with new approaches to engage society in IAS control (e.g. citizen science, Clusa et al. 2018) and for biomonitoring (e.g. DNA metabarcoding, Borrell et al. 2017). In addition, risk assessments and horizon scanning studies for decision-making formed a notable part of the literature, with direct implications for IAS regulation (Bayón and Vilà 2019). There was also discussion regarding the inefficiency of current codes of conduct and laws (Maceda-Veiga et al. 2013), aimed at enforcing policies to overcome the highlighted deficiencies.

The proportion of applied studies and field experiments was markedly low, which impeded rating the efficacy of most of the described approaches (66.2%). Moreover, studies on novel biocontrol agents, such as the use of pathogens (McColl and Sunarto 2020) were infrequent. In part, this is because these agents require rigorous risk assessment studies and are, hence, subject to strict regulations (Loomans 2021). Thus, species eradication and control experiences often relied on the use of herbicides, mechanical elimination or culling (e.g. Melero et al. 2010; Mateos-Naranjo et al. 2012) and successful approaches were restricted to small-scale areas, such as islets and ponds (e.g. Ferreras-Romero et al. 2016; Maceda-Veiga et al. 2017). Consequently, concerns of Spanish managers about the problem that too much research focuses generally on the ecological aspects of alien plants, rather than on specific cost-efficient management strategies (Andreu et al. 2009), can be considered, to some extent, applicable to most invasive alien taxa.

The proportion of documents per species and ecosystem type in Spain was similar to that estimated in other countries (Thomsen et al. 2014). Terrestrial species, mainly plants, attracted the bulk of the literature, followed by studies involving species of inland waters. By contrast, marine and urban environments were infrequent in the collected literature. The preponderance of terrestrial ecosystems and the associated species can be justified by the primary introduction pathways of terrestrial species (i.e. release and/or escape), which are largely related to forestry, livestock, agriculture and wildlife trade (Essl et al. 2015). Vascular plants are the most frequently introduced taxon worldwide; consequently, they were expected to receive the largest proportion of studies. However, the feasibility of managing terrestrial invaders or aliens, especially sessile species and stages of their life history (e.g. nests, Enríquez et al. 2013), is greater compared to aquatic species, due to the lower accessibility of these environments. Therefore, a publication bias towards successful studies with positive results on tractable terrestrial species is not discernible (Fanelli 2012; Booy et al. 2017).

Studies on terrestrial ecosystems involved all spatial scales (i.e. local, regional, national and supranational). However, applied experiments and experience were mostly local interventions, such as management of prickly pear species (*Opuntia* spp.) and the sentry plant (*Agave americana*) (Arévalo et al. 2015). By contrast, eradication and control of vagile terrestrial organisms proved to be economically unaffordable (e.g. American mink *N. vison*, Melero et al. 2010; Mañas et al. 2016), especially in a context of multilevel overlapping or competing public administrations with ill-defined jurisdictions and pervasive budget shortages (Tollington et al. 2017; Dana et al. 2019). In this regard, island territories were well covered by the collected literature, with several successful management experiences in these territories, such as the eradication of American mink (*N. vison*) in the Atlantic Islands National Park (Velando et al. 2017) or of the red palm weevil (*R. ferrugineus*) from the Canary Islands (Fajardo et al. 2019). Impacts of IAS on islands are likely to increase in the future, especially on oceanic islands, such as the Canaries and, to a lesser extent, on the continental Balearic Archipelago. Indeed, insular terrestrial ecosystems are generally the most threatened (Lenzner et al. 2020). Therefore, these two successful examples shed hope on the future management of IAS in Spanish insular territories.

The published research on terrestrial invertebrates and microorganisms appeared to be concentrated on a few species with direct impacts on economics and human health (e.g. yellow-legged hornet *V. velutina*, pinewood nematode *B. xylophilus* or tiger mosquito *A. albopictus*). However, further applied research and knowledge transfer is particularly needed to control invertebrates, due to the rising number of introductions worldwide (Saul et al. 2017; Seebens 2019). Such a task often requires strategies based on prevention and prompt eradication (e.g. ballast water sanitation) (Booy et al. 2017, 2020), but these were the least frequent of the categories amongst those implying direct manipulation of the target taxa. From a theoretical viewpoint, the compiled literature raises awareness and proposes changes to current regulations as its main approach, directed at preventing further introductions at a higher level of organisation. Legislation regarding IAS has become more restrictive over the years worldwide and Spain is also immersed in this useful trend (Turbelin et al. 2017; Maceda-Veiga et al. 2019). However, in light of the number of recent introductions (Muñoz-Mas and García-Berthou 2020), it can be concluded that the real effective capability of Spain to impede the establishment of further species is limited. This general pattern is shared with other European countries and is unlikely to change substantially in the near future (Seebens et al. 2021). Tackling the establishment of further terrestrial invertebrates and microorganisms will require further and stronger innovative and well-funded preventative approaches.

The number of studies conducted in inland waters was notably high due to the enormous number of established species and their associated economic costs (e.g. Durán et al. 2012; Muñoz-Mas and García-Berthou 2020). Some of this research was promoted by the Water Framework Directive (European Parliament & Council 2000), even if IAS are not explicitly mentioned therein (Boon et al. 2020). Our keyword co-occurrence network reflected the numerous studies recommending flow management as a way to control IAS (Sabater et al. 2008; Fornaroli et al. 2020). However, despite the schemes conducted in other countries (Kiernan et al. 2012), no applied examples in large and intermediate regulated river systems were found in the literature. River basin management plans increasingly account for the presence of IAS, but more emphasis on applied management of medium-to-large river systems is necessary (Boon et al. 2020). Nonetheless, applied studies of inland water ecosystems dealt with control of the giant reed *A. donax* (the most managed species appearing in the compiled literature) in relatively small areas (Bruno et al. 2019) or described experience in relatively small lentic environments (i.e. common carp *Cyprinus carpio* in ponds and lakes; Ferreras-Romero et al. 2016) and small streams (signal crayfish Pacifastacus leniusculus; Dana et al. 2010). Unfortunately, more research is needed to optimise water allocation schemes because climate change is facilitating the establishment of further IAS. Meanwhile, the increased demands of agriculture will reduce the availability of water resources to undertake the aforementioned actions (Rahel and Olden 2008; Escribano Francés et al. 2017).

In Spain, stowaway introductions in brackish and marine environments have also gained prominence (García-Gómez et al. 2020; Painting et al. 2020), causing a shift in the type of introduced species that can be framed within the current increase in global maritime traffic (Saul et al. 2017; Seebens 2019). In this regard, Spain enacted in 2004 the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention), which has been addressed in local studies (e.g. Moreno-Andrés et al. 2017), dissertations (e.g. Bartolomé Lamarca 2014) and is highlighted in the keyword co-occurrence network. However, new IAS records are being reported frequently. For example, the gastropod *Mitrella psilla* was recently found thriving on western coasts of Spain (Martínez-Ortí et al. 2020). The effectiveness of measures to fulfil the BWM Convention remains limited, which underscores the difficulties faced in managing marine IAS (Thomsen et al. 2014; Cuesta et al. 2016). Indeed, the number of first records whose most probable introduction pathways are ballast waters or biofouling (Davidson et al. 2018) and the increasing importance of aquaculture and related introductions (Nunes et al. 2015; Garlock et al. 2020) suggest these introduction pathways must be taken into account. It can, therefore, be concluded that marine invasion science should move towards our central focus in the future.

Worldwide, urban environments are becoming active introduction hubs (Gaertner et al. 2017). Indeed, there are several examples of initial IAS establishment in urban zones that have spread outwards over natural environments in Spain, such as *Lippia filiformis* (Casasayas i Fornell 1989) or the black-headed weaver *Ploceus melanocephalus* (Grundy et al. 2014). Ornamental plants and alien exotic birds kept as pets are perhaps the most striking and troublesome introductions in city surroundings (Riera et al. 2021), but other less-known taxa have been found on numerous occasions (e.g. *Pseudosuccinea columella* (Mollusca) Martínez-Ortí 2013). Besides the introduction of ornamental plants and tortoises and terrapins (*Trachemis* spp.), which have already spread over natural environments and prompted specific studies (Muñoz-Mas and García-Berthou 2020), the majority of Spanish management literature on urban environments has focused on birds (e.g. the monk parakeet *Myiopsitta monachus* and the rose-ringed parakeet *Psittacula krameri* (Álvarez-Pola and Muntaner 2009; Maceda-Veiga et al. 2019; Hernández-Brito et al. 2020; Saavedra and Medina 2020). Therefore, in the light of increasing urbanisation of the Spanish population and the relatively low number of specific studies, we conclude that prevention protocols and further studies, specifically addressed to urban environments, should be strengthened to encompass the full spectrum of potential introductions.

The compiled literature on IAS management in Spain does not particularly reflect budget reductions related to the Great Recession of 2008 (Catanzaro 2018). However, IAS management literature has been under-represented compared to overall scientific production trends and the current rise in publication rates has not compensated for this historical delay. Nonetheless, our compiled references only represent a small fraction of the total number of reviewed documents (388/3796 = 10.22%). Moreover, a significant number of documents were written in Spanish (n = 158; 40.7%). This finding deserves special attention as it handicaps knowledge transfer (Di Bitetti and Ferreras 2017) and biases conclusions inferred by international agents (Konno et al. 2020). However, it is not problematic from a national point of view because texts, documents and software packages assisting the process of decision-making by administrators and functionaries have proven to be most efficient when presented in local languages (Copp et al. 2021). Nevertheless, despite the proliferation of public repositories and open access publications, a wealth of information is still hidden away, not easily accessible to risk assessors, managers and researchers through standard search engines. For example, it is known that more than a hundred plant species have been managed by Spanish regional administrations (Andreu and Vilà 2007), but only the most frequent species generated accessible documents (e.g. reports). Altogether, it indicates that public agencies produce insufficient literature (sensu lato) as they focus on other tasks. Neither communication between managers and scientists beyond undertaking management action schemes nor protocols to evaluate their success are common practices in Spain. It would be beneficial for public agencies to encourage and facilitate such interguild contact, perhaps using legislative and labour changes, to disseminate applied experience in accessible ways.

Despite these recommendations and the highlighted deficiencies, Spanish literature on IAS management should not be considered completely defective. Recent studies on alien animal species, currently thriving in Spanish inland waters, indicate that no single management protocol can be applied to every taxonomic group, due to marked differences amongst species, introduction pathways and invaded habitats (Muñoz-Mas and García-Berthou 2020). Likewise, our study shows a strong association between species, taxon or group of taxa and the features/categories used to describe the compiled literature. This indicates that species-specific studies are often needed, which highlights how difficult and complex the task of IAS management is (Woodford et al. 2016; Portela et al. 2020; Yelenik et al. 2020). Our results should help to properly drive future research efforts towards IAS management in Spain. We recommend more research into applied techniques to shift the balance between theoretical and empirical studies, especially in inland waters and marine ecosystems due to their lower accessibility. The same need for more studies applies to urban environments, as they are often the bridgehead of IAS introductions. Renewed effort in prevention and prompt eradication should be made to fulfil, for example, the BWM Convention and impede further introductions into marine ecosystems. Finally, we encourage public agencies to support and strengthen the dissemination of applied experience and thus enhance know-how and knowledge transfer in the field.

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Supplementary material 1

Complete reference list and the features used to characterise the references

Authors: Rafael Muñoz-Mas, Martina Carrete, Pilar Castro-Díez, Miguel Delibes-Mateos, Josep A. Jaques, Marta López-Darias, Manuel Nogales, Joan Pino, Anna Traveset, Xavier Turon, Montserrat Vilà, Emili García-Berthou.

Data type: references and features

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Supplementary material 2

Table S1. Table of features, categories and definitions used to characterise the compiled literature

Authors: Rafael Muñoz-Mas, Martina Carrete, Pilar Castro-Díez, Miguel Delibes-Mateos, Josep A. Jaques, Marta López-Darias, Manuel Nogales, Joan Pino, Anna Traveset, Xavier Turon, Montserrat Vilà, Emili García-Berthou

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RESEARCH ARTICLE

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Current limitations and future prospects of detection and biomonitoring of NIS in the Mediterranean Sea through environmental DNA

Francesco Zangaro[1,2*], Benedetta Saccomanno[1*], Eftychia Tzafesta[1],
Fabio Bozzeda[1,2], Valeria Specchia[1], Maurizio Pinna[1,2]

1 *Department of Biological and Environmental Sciences and Technologies, DiSTeBA, University of Salento, via Monteroni 165, 73100 Lecce, Italy* **2** *Research Centre for Fisheries and Aquaculture of Aquatina di Frigole, DiSTeBA, University of Salento, 73100 Lecce, Italy*

Corresponding author: Maurizio Pinna (maurizio.pinna@unisalento.it)

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Abstract

The biodiversity of the Mediterranean Sea is currently threatened by the introduction of Non-Indigenous Species (NIS). Therefore, monitoring the distribution of NIS is of utmost importance to preserve the ecosystems. A promising approach for the identification of species and the assessment of biodiversity is the use of DNA barcoding, as well as DNA and eDNA metabarcoding. Currently, the main limitation in the use of genomic data for species identification is the incompleteness of the DNA barcode databases. In this research, we assessed the availability of DNA barcodes in the main reference libraries for the most updated inventory of 665 confirmed NIS in the Mediterranean Sea, with a special focus on the cytochrome oxidase I (COI) barcode and primers. The results of this study show that there are no barcodes for 33.18% of the species in question, and that 45.30% of the 382 species with COI barcode, have no primers publicly available. This highlights the importance of directing scientific efforts to fill the barcode gap of specific taxonomic groups in order to help in the effective application of the eDNA technique for investigating the occurrence and the distribution of NIS in the Mediterranean Sea.

Keywords

Biomonitoring, COI, DNA barcode, environmental DNA – eDNA, gap analysis, Mediterranean Sea, Non-Indigenous Species – NIS, reference libraries

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* Those authors contributed equally to this work.

Introduction

The Mediterranean Sea represents one of the most important biodiversity hotspots in the world (Myers et al. 2000; Coll et al. 2010; Lejeusne et al. 2010; Marrocco et al. 2019), accounting for more than 17,000 reported marine species (Coll et al. 2010). However, the number of Non-Indigenous Species (NIS) and their impact on native species is steadily increasing (Villèle and Verlaque 1995; Streftaris and Zenetos 2006; Marrocco et al. 2018; Bariche et al. 2020). Therefore, the Mediterranean Scientific Community highlights the importance of early warnings and monitoring the presence and distribution of NIS (Katsanevakis et al. 2016; Darling et al. 2017; Tsiamis et al. 2020). Nowadays, this is a key requirement for the conservation and management of ecosystems, as stated by the regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species (Tiralongo et al. 2019).

Until now, the assessment of Mediterranean species diversity has been carried out through traditional methods based on sole morphological identification. These methods present several disadvantages, such as the difficulty in surveying large geographical areas and spotting and identifying the so-called "hard-to-detect species" (Tiralongo et al. 2020). Besides, they are mainly based on recognizable adult features, such as the shape of gonads or other particular body parts and, often, do not give any identification key for the larval forms or the early developmental stages (Ponti et al. 2009; Di Sabatino et al. 2014; Pinna et al. 2017). They also easily mislead the identification of individuals when their morphology is altered by stressful environmental conditions or by sampling and preservation techniques (Leese et al. 2016; Pawlowski et al. 2018; Tiralongo et al. 2020). In addition, traditional phenotypic-based methods require the expertise of taxonomists, especially when there is the need to identify a species never observed before in a certain area, including NIS (Leese et al. 2016; Pawlowski et al. 2018).

Consistent biological records can provide a better understanding of the distribution of marine species, their expansion range, and the arrival of new NIS in the Mediterranean basin (Mannino et al. 2019; Bariche et al. 2020). A promising approach for the identification of species and biomonitoring of ecosystems is the use of molecular tools such as DNA barcoding, metabarcoding and environmental DNA (Pawlowski et al. 2018; Specchia et al. 2020; Pinna et al. 2021; Tzafesta et al. 2021). DNA barcoding refers to a single species identification with the use of a short DNA fragment, while in the metabarcoding technique the DNA is extracted from a sample containing more than one organism/species, amplified and sequenced by Next Generation Sequencing (NGS) (Ji et al. 2013; Deiner et al. 2017). DNA metabarcoding allows the identification of species at low densities and the detection of taxa that traditional approaches generally fail to distinguish (Pawlowski et al. 2018; Zangaro et al. 2020). Another innovative technique for the identification of species at low concentration is the eDNA (Pawlowski et al. 2018), which is based on the extraction of DNA directly from environmental samples like water or sediment (Rees et al. 2014). This technique can efficiently be applied for assessing the presence and the distribution of NIS that are hard to detect.

Also, it may result in a perfect tool for monitoring and preventing new NIS arrivals, for instance, by analysing the genetic content of ballast waters and monitoring it in water exchanges between different regions (Bariche et al. 2020; Tzafesta et al. 2021).

However, even the application of molecular techniques faces some challenges. The level of uncertainty linked to eDNA for marine environments depends generally on the persistence time of the DNA in marine systems (Collins et al. 2018), the high level of connection and movement due to the aquatic medium, and the incompleteness of the reference public libraries (Cagnacci et al. 2012; Weigand et al. 2019; Specchia et al. 2020). Once a DNA fragment has been sequenced, it needs to be blasted into reference libraries to identify the species it belongs to. Moreover, the success of the eDNA technique is also based on the efficiency of the primer sets on large numbers of taxa, a key requirement to correctly amplify the investigated gene in the environmental sample to identify as many species as possible (Elbrecht et al. 2017).

The main DNA barcode reference libraries are GenBank, by the National Centre for Biotechnology Information (NCBI), and BOLD (Barcode of Life Data) Systems (Ratnasingham et al. 2007; Leese et al. 2016; Macher et al. 2017). The information available in the reference libraries includes the species name, the nucleotide sequence of the target genes and, optionally, the PCR primer pairs used for the amplification of the gene of interest in the target organism (Ratnasingham et al. 2007; Macher et al. 2017).

The Consortium for the Barcode of Life (CBOL; www.barcoding.si.edu.com) and the International Nucleotide Sequence Database Collaborations (INSDC) designated the mitochondrial cytochrome oxidase subunit I (COI) as the main barcoding gene based on its widespread presence among different taxonomic groups (Hebert et al. 2004a; Hebert et al. 2004b; Saunders 2005; Ward et al. 2005). Moreover, nucleotide sequence polymorphisms of this approximately 500 bp COI barcode region provide valuable information not only on species identification but also on population genetic diversity and structure (Goetze et al. 2016; Abbas et al. 2018; Choo et al. 2020). Several barcoding studies have also identified alternative genes that can be successfully used for molecular barcoding and may be more suitable to a specific taxonomic group. For example, the ribosomal genes 16s and 18s are generally used for the identification of prokaryotes (Stackebrandt 1994; Acina et al. 2004) and eukaryotes (Hadziavdic et al. 2014; Bradley et al. 2016), respectively; the nuclear ribosomal internal transcribed spacer 1 and 2 (ITS) for fungi (Scoch et al. 2012; Badotti et al. 2017); and two plastid genes, the maturase-coding gene (matK) and the large subunit of ribulose 1,5-bisphosphate carboxylase-coding gene (rbcL) for plants (CBOL 2009), among others.

In light of this, we wanted to evaluate the current status of DNA barcode availability for the NIS already detected in the Mediterranean through morphological surveys. To do so, we retrieved the most recent list of NIS published by Zenetos and Galanidi (2020) and we looked for the availability of COI barcodes and primers in reference libraries. If COI barcodes were not retrieved, we then searched for other barcoding genes. If no records were found in the reference libraries, this was then referred to as DNA barcode gap.

The aim of this research is to evaluate the current limitations in the application of molecular barcoding due to the barcode gap of Mediterranean NIS, and to investigate in depth the occurrence of COI gene barcode and primer pairs. Furthermore, we indicate which taxonomic groups may be underestimated by using molecular tools for the detection and biomonitoring of NIS in the Mediterranean Sea through environmental DNA.

Materials and methods

Checklist of NIS occurring in the Mediterranean Sea

We obtained an updated checklist of confirmed alien species occurring in the Mediterranean Sea using an inventory of NIS published by Zenetos and Galanidi at the start of 2020. In this inventory, a total of 666 marine NIS established in the Mediterranean Sea are divided into 10 high-ranked taxonomic groups, as defined by the authors (Zenetos and Galanidi 2020).

The names of the species were verified using the following platforms: EU-NOMEN (http://www.eu-nomen.eu), FishBase (https://www.fishbase.de), ALGAEBASE (https://www.algaebase.org), EASIN (https://easin.jrc.ec.europa.eu/easin) and WORMS (http://www.marinespecies.org) (Specchia et al. 2020). This resulted in a total number of 665 NIS because we excluded *Chaetoceros bacteriastroides*, an uncertain (unassessed) species on all the above-mentioned platforms. For each of the 665 NIS, we considered the currently accepted name and all of the synonyms and older names, to ensure that the species in question is really absent in the reference libraries.

DNA barcode libraries interrogation and data analysis

The 665 NIS official and alternative nomenclatures were manually entered in BOLD Systems and GenBank to search for a COI barcode. If a COI barcode was retrieved, we then looked into the availability of primer pairs and their use across different taxonomic groups. We also recorded other genes (5.8s, 12s, 16s, 18s, 28s, cytb, rbcL), when the COI barcode was not available, to correctly estimate the barcode gap. All the data was compiled in an Excel file available as Suppl. material 1: Table S1, which we used as a starting point to quantify the barcode gap as a percentage of species within each group.

Results

COI barcode and primer gap in NIS occurring in the Mediterranean Sea

In total, 665 NIS established in the Mediterranean Sea, belonging to 132 orders, were divided into 10 main taxonomic groups (Fish, Parasites, Phytobenthos, Ascidians, Bryozoa, Crustacea, Miscellanea, Mollusca, Polychaeta, and Zooplankton; Zenetos and Galanidi 2020), and their DNA barcoding gap in reference libraries was investigated. At the end of June 2021, 220 out of 665 NIS did not have any barcodes in reference libraries (BOLD Systems and GenBank), showing a barcoding gap of 33.18%. Of the remaining 445 barcoded species, 14.16% did not have a COI barcode but still presented another gene barcode (Suppl. material 1: Table S1).

For the 382 species associated with a COI barcode in the DNA reference libraries, we further looked into the availability of primer pairs, finding that 45.30% do not have publicly available primer pairs. Moreover, of the 55 primer pairs found across different taxonomic groups, only 4 pairs were used in more than one phylum. They are LCO1490/HCO2198, LCO1490_t1/HCO2198_t1 and C_LepFolF/C_LepFolR found in Chordata, Arthropoda and Mollusca, and jgLCO1490/jgHCO2198 found in Arthropoda, Chordata, Mollusca, Bryozoa and Echinodermata. No universal primer pairs were identified.

Barcode and primer pair gaps in taxonomic groups

In the "Bryozoa" group, 30 NIS, divided into 2 orders, have been analysed. Among these, 23 species (76.67%) were not associated with a DNA barcode (Fig. 1), representing the group with the largest gap. Six species present a COI barcode, while only one (*Celleporella carolinensis*) does not have any public record apart from a partial coding DNA sequence (cds) of the elongation factor 1 alpha. For this taxonomic group, only 1 primer pair (jgLCO1490/jgHCO2198) for COI gene amplification was identified and it is only used in two species of the same genus: *Celleporaria aperta* and *Celleporaria brunea.*

The second group with the most extensive barcode gap is represented by "Parasites", consisting of 25 NIS divided into 10 orders. In this group, 14 species (56%) lack a barcode, 8 (32%) have a COI barcode and 3 (12%) have a different gene barcode (Fig. 1). The 3 species lacking COI have a record on GenBank of a coding sequence (cds) annotated as ribosomal subunit, which we identified through BlastN as 28s for *Boninia neotethydis* and *Tetrancistrum polymorphum*, and 18s for *Thulinia microrchis.*

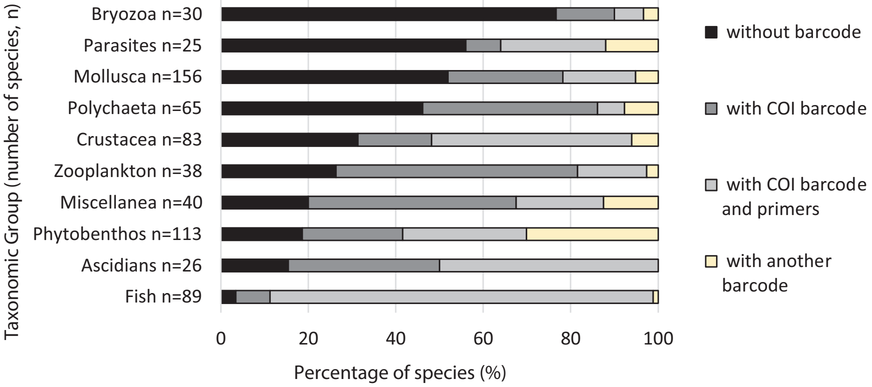

Figure 1. DNA Barcode gap of Mediterranean NIS. Data are presented as a percentage of the total number of species in each group (n).

Like Bryozoa, Parasites display a substantial COI primer pair gap, having only 2 sets of primers available: HCO2198/LCO1490, only used in *Heterosaccus dollfusi* and jgHCO2198/jgLCO1490, only used in *Livoneca redmanii.*

The group "Mollusca" contains the highest number of NIS, amounting to 156 species divided into 28 orders. Among these, 81 species (51.92%) are not associated with a DNA barcode, 67 (42.95%) have a COI barcode, while 8 (5.13%) have another barcode, mainly represented by 16s, 18s and 28s (Fig. 1). For this taxonomic group, 13 different COI primer pairs have been identified. These primer pairs were tested in 26 species, leaving a COI primer pair gap in 41 species (26.81%).

The group "Polychaeta" consists of 65 NIS, divided into 7 orders. Among these, 30 species (46.15%) are not associated with a DNA barcode, 30 (46.15%) have a COI barcode and 5 (7.69%) have other barcodes, represented by the following genes: 5.8s, 16s, 18s, 28s and cytochrome b (cytb). This taxonomic group shows the largest COI primers pair gap, covering 86.67% of COI barcoded species. Only four primer pairs were found: jgLCO1490/jgHCO2198 used in *Branchiomma bardi* and *Timarete punctata*; polyLCO/polyHCO and C_VF1LFt1/C_VR1LRt1 both used in *Pileolaria berkeleyana*; and mlCOIintF/HCO2198 only used in *Polydora cornuta.*

The group "Crustacea" consists of 83 NIS, divided into 7 orders. Among these, 26 species (31.33%) are not associated with a DNA barcode, 52 species (62.65%) have a COI barcode and 5 species (6.02%) have another barcode. Four out of these 5 species lacking COI present either 12s, 16s or both, while one (*Thalamita poissonii*), presents Thapmar 1.5 transposon as the only record. For this taxonomic group, 9 different primer pairs for COI amplification were found. These 9 primers were used in 38 species (45.78%), while the remaining 14 species with COI sequence did not have a primer set, resulting in a COI primer pair gap of 16.87%. The most used primer set is HCO2198/LCO1490, found in 26 out of 38 species (68%).

The group "Zooplankton" consists of 38 NIS divided into 14 orders. Among these, 10 (26.32%) are not associated with a DNA barcode, 27 (71.05%) have a COI barcode and only one (*Parvocalanus elegans*) does not have COI but 28s, instead. This group is the one with the second largest COI primer pair gap, having 4 primer sets used only on 6 out of 26 COI barcoded species, giving a COI primer gap of 77.78%.

The group "Miscellanea" consists of 40 NIS divided into 6 phyla and 21 orders. Among these, 8 species (20%) are not associated with a DNA barcode, 27 (67.50%) have a COI barcode and 5 (12.50%) have either 16s, 18s, or both. For this taxonomic group, eight primer pairs were found, but used only on 8 out of 27 COI barcoded species, leaving a primer pair gap of 70.37%.

The group "Phytobenthos" is the second-largest NIS group, with 113 NIS divided into 27 orders. Among these, 21 species (18.58%) are not associated with a DNA barcode, 58 species (51.33%) have a COI barcode and 34 species (30.09%) have another barcode. This is the group where an alternative barcode gene to COI has been used the most since COI is generally used for barcoding animal species. RbcL is the most represented gene for this group, covering 25 out of 34 species. For this taxonomic group, a total of 12 primer sets for COI amplification were found. These primers were used on

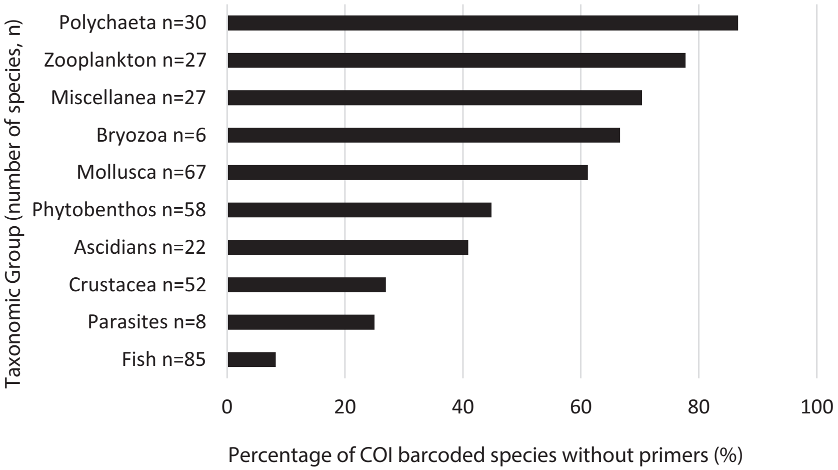

Figure 2. Primers not available in COI barcoded species. Data presented as a percentage of the total number of COI barcoded species

26 out of 58 species, leaving a COI primer pair gap of 44.83% (Fig. 2). The most used primer set is GWSFn/GWSRx, present in 17 out of 26 species (65.38%).

The group "Ascidians" consists of 26 NIS divided into 3 orders. Among these, 4 species (15.38%) are not associated with a DNA barcode and 22 species (84.62%) have a COI barcode. No other barcoding genes were found. Five primer sets were identified, covering 13 out of 22 barcoded species, leaving a primer pair gap of 40.91%. The most used primer sets are jgLCO1490/jgHCO2198 and Tun_Forward/Tun_reverse2, used in 6 and 7 species, respectively.

The group "Fish" consists of 89 NIS divided into 15 orders. Among these, only 3 (3.37%) species are not associated with a barcode, 86 species (96.63%) have a COI barcode and 1 (*Caesio varilineata*) does not have a COI barcode, but a 12s, instead (Fig. 1). For this taxonomic group, 19 different primer pairs for COI gene amplification have been identified. These 19 primers cover a total of 78 species, leaving only 8.24% of COI barcoded species without a primer set (Fig. 2). The most used primer sets are C_FishF1t1/C_FishR1t1 and VF2/VR1, found in 54 and 51 species respectively, 34 of which present both primers.

Discussion

The aim of this study was to quantify the extent of the DNA barcode gap for the NIS established in the Mediterranean Sea, as identified by Zenetos and Galanidi in 2020 and, in doing so, to direct the efforts of the scientific community towards specific taxonomic groups. The data show that 33% of NIS do not have any record in public libraries, making it impossible to detect these species through DNA barcoding techniques. Bryozoa and Parasites are the ones with the largest gap relative to the total number of species in each group. However, Mollusca, which covers almost 25% of the total number of NIS, also needs attention having a barcode gap in 50% of the species, followed by Polychaeta with a gap of 41%. On the other hand, Phytobenthos, Ascidians and especially Fish appear to be the groups that could be mostly identified through molecular techniques, having smaller barcode gaps of 18%, 14% and 3%, respectively.

Our analysis highlighted the importance of analysing barcode gaps in reference libraries for the successful application of molecular tools (including eDNA and DNA metabarcoding) in biomonitoring assessments. Gap-analysis surveys focusing on DNA barcode presence in public repositories for different groups of species are recently gaining greater attention from the scientific community. Gap-analysis has already been applied on marine NIS (Duarte et al. 2021), on macrofauna of a region of the North Sea (Hestetun et al. 2020), on aquatic macroinvertebrates of South-East Italy (Specchia et al. 2020), on marine macroinvertebrates of the Atlantic Iberia (Leite et al. 2020), and on Ascidians and Cnidarians of the European Register of Marine Species (ERMS; Paz and Rinkevich 2021). However, to our knowledge, this is the first study that investigates the DNA barcode gap for NIS occurring in the Mediterranean Sea.

This study also confirms that COI is a useful genetic marker because it is broadly sequenced across different phyla, making it a good candidate gene for identifying species in an environmental sample. Nonetheless, relying only on one DNA fragment may lead to misidentification of pooled samples due to possible sequence similarity; this is why multigene approaches should be preferred in molecular biomonitoring studies (Zou et al. 2012; Chesters et al. 2015; Gangan et al. 2019). Phytobenthos could be the first group to apply this approach, having 30% of species already barcoded with rbcL but not with COI. This is probably explained by the fact that rbcL is a standard barcode for plants (CBOL 2009; Maloukh et al. 2017; Kang et al. 2017; Weigand et al. 2019), further proving the advantage of selecting not only universal barcodes but also relevant taxa-specific genes.

Moreover, the success of the eDNA metabarcoding is based on the availability of efficient primer sets for the amplification of several taxa in a given sample (Elbrecht et al. 2017; Tzafesta et al. 2021). However, only 26% of NIS occurring in the Mediterranean Sea appear to have publicly available COI primers. Especially for animals, 147 species out of 324 COI barcoded species (45.40%) were lacking primer pairs, which highlights the need for further evaluation of primers or the design of new ones. In addition, no universal primer pair was identified, resulting in more laborious molecular identifications where an environmental sample needs to be amplified with several sets of taxa-specific or even species-specific primers to be correctly assessed.

For the above reasons, also the primer pair gap needs to be filled. In order to do so, both increasing the surveys regarding NIS occurring in the Mediterranean Sea and improving the barcoding studies at a global scale is essential, as well as biodiversity assessments (Zangaro et al. 2021). Although the content of the databases doubles approximately every 18 months (https://www.ncbi.nlm.nih.gov/genbank/statistics/), probably many of the NIS established in the Mediterranean Sea come from underdeveloped regions, which cannot financially support molecular surveys.

This is easily extrapolated by looking at the "Data Releases" page provided by BOLD Systems (https://www.boldsystems.org/index.php/datarelease). Because of that, we not only encourage collaboration of researchers in this sector, but we also stress the need for training and inclusion of researchers from developing countries, which represent the current and, probably, the future source of new and hard-to-detect NIS.

In conclusion, it is essential to underline that molecular techniques represent a great opportunity to improve the study on the occurrence and distribution of NIS. Hence, a specific gap needs to be filled by the scientific community to make molecular identification totally efficient and independent at a regional, national, and transnational level.

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Supplementary material 1

Table S1

Authors: Francesco Zangaro, Benedetta Saccomanno, Eftychia Tzafesta, Fabio Bozzeda, Valeria Specchia, Maurizio Pinna

Data type: excel file

Explanation note: COI PCR primer pairs.

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Establishment and new hosts of the non-native seed beetle *Stator limbatus* (Coleoptera, Chrysomelidae, Bruchinae) on acacias in Europe

Arturo Cocco[1], Giuseppe Brundu[1], Cyril Berquier[2,6],
Marie Cécile Andreï-Ruiz[2,6], Michelina Pusceddu[1], Marco Porceddu[3,4],
Lina Podda[3,4], Alberto Satta[1], Yohan Petit[5,6], Ignazio Floris[1]

1 *Department of Agricultural Sciences, University of Sassari, Viale Italia 39, Sassari, Italy* **2** *Observatoire Conservatoire des Insectes de Corse (OCIC), 14 Avenue Jean Nicoli, Corte, France* **3** *Department of Life and Environmental Sciences, Centre for the Conservation of Biodiversity (CCB) University of Cagliari, Viale S. Ignazio da Laconi 13, Cagliari, Italy* **4** *Sardinian Germplasm Bank (BG-SAR), Hortus Botanicus Karalitanus (HBK), University of Cagliari, viale S. Ignazio da Laconi 9-11, Cagliari, Italy* **5** *Conservatoire Botanique National de Corse (CBNC), 14 Avenue Jean Nicoli, Corte, France* **6** *Office de l'Environnement de la Corse (OEC), 14 Avenue Jean Nicoli, Corte, France*

Corresponding author: Arturo Cocco (acocco@uniss.it)

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Abstract

Stator limbatus is a phytophagous beetle native to warm regions of North and Central America, feeding on Fabaceae seeds and one of the most polyphagous species within the subfamily Bruchinae, here reported for the first time in Europe and on new hosts. Adult beetles emerged from *Acacia* spp. seeds collected in the islands of Corsica (France), and Sardinia (Italy). The wide presence in Sardinia and Corsica supports the hypothesis that this alien species was introduced several years ago. In both islands, *S. limbatus* emerged from *Acacia mearnsii* seeds, with infestation rates of up to 74.2 and 90.8% in 2019 and 2020, respectively. This seed beetle also emerged from two previously unreported host species, *Acacia saligna* and *A. pycnantha*, showing highest infestation rates of 4.0 and 95.1%, respectively. Both *Acacia* species are reported as new host associations with *S. limbatus*. Overall, seed infestation rates recorded in 2019 and 2020 indicate that *S. limbatus* is well established and that Mediterranean bioclimatic conditions are suitable for its population increase in size. This study lays the foundations for further research on known and potential host species and the spread and distribution of *S. limbatus* in Europe.

Keywords
Acacia mearnsii, *Acacia pycnantha*, *Acacia saligna*, alien species, bean weevil, biological invasion, Mediterranean islands

Introduction

The global movement of people and goods and climate change are dramatically promoting the introduction of alien species in non-native environments in the Anthropocene (Kueffer 2017), resulting in a continuous accumulation of these species worldwide (Seebens et al. 2017; Venette and Hutchison 2021). This indicates that current measures to avoid new introductions of alien species are not always effective. Therefore, prevention, continuous monitoring in priority sites, early detection, and rapid intervention are of major importance for avoiding the establishment of new invasive alien species and agricultural or forestry pests and for reducing the spread of the existing ones, with special concern towards protected areas and natural ecosystems.

Among seed-feeding insects, the subfamily Bruchinae (Coleoptera, Chrysomelidae) beetles, renowned as bean weevils, is highly specific and likely the most important (van Klinken 2005). This family includes about 4,350 taxa distributed worldwide (Borowiec 1987). The beetle *Stator limbatus* (Horn, 1873) (Coleoptera, Chrysomelidae: Bruchinae) is an endophagous seed feeder of legumes (Fig. 1). Its native range spans from semiarid and xeric regions of southwestern United States and northern Mexico to dry tropical forests of Central America and northern South America. *Stator limbatus*

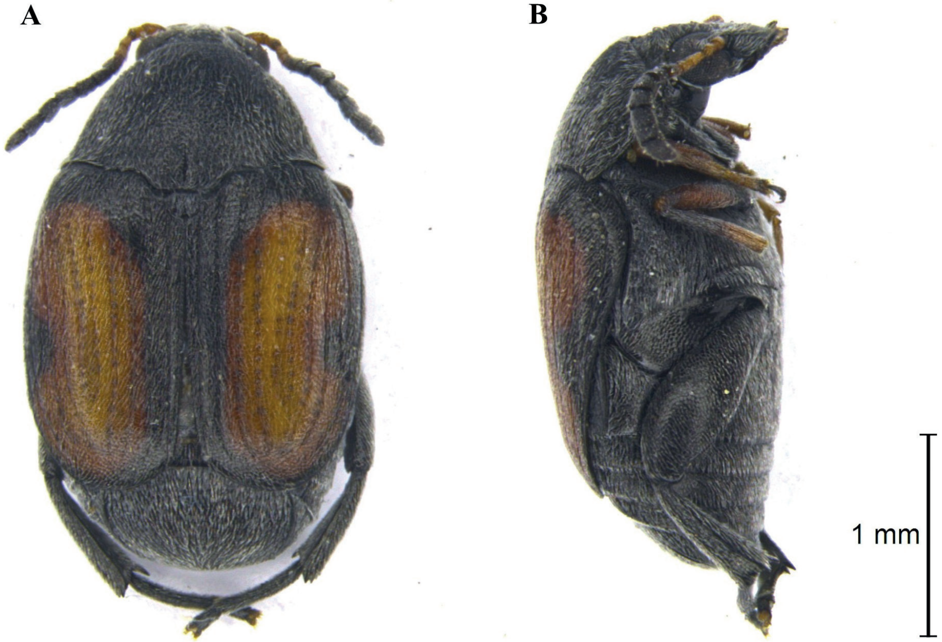

Figure 1. Habitus of adult *Stator limbatus* **A** dorsal and **B** lateral view.

has a generalist habit and a wide host range, as it has been collected from > 90 host plant species (de Jesús Parra-Gil et al. 2020), including many species of the genus *Acacia* s.l. In its native range, it affects mostly native species, but also about 20 non-native species (Stillwell et al. 2007). Despite that, host colonization of *S. limbatus* populations varies greatly among regions, and distinct populations exhibit host specialization at a local scale (Morse and Farrell 2005a, 2005b). Beetle populations are known to express phenotypic plasticity to host species by adapting pre-imaginal development time and body and egg size (Amarillo-Suarez and Fox 2006; Amarillo-Suarez et al. 2017).

Eggs are oviposited on mature seeds inside of dehiscent or partially dehiscent pods when they are still on the plant (Johnson 1981a; Kingsolver 2004). Females usually lay one egg per seed, and newly hatched larvae burrow into the seed integument beneath the egg, complete their development and pupate inside the same seed. In the case in which seeds are limiting, more eggs are deposited across a seed (Morse and Farrell 2005a). Beetles emerge from seeds as adults, mate and females start ovipositing within 24–48 hours, under laboratory conditions. Adults are facultatively aphagous, as they only require resources acquired during the pre-imaginal stage to complete development and reproduce (e.g. capital breeders) (Stillwell and Fox 2009). The generation time at 28 °C was determined to be 28–30 days (Amarillo-Suarez and Fox 2006).

Several species within the *S. limbatus* host range, such as *Acacia mearnsii* De Wild and *Acacia saligna* (Labill.) H.L.Wendl. native to Australia, have shown in Europe invasive potential and negative impacts on native species, to the extent that containment measures have been implemented (Lowe et al. 2000; European Union 2014; Tozzi et al. 2021). Therefore, monitoring the presence of seed beetles of invasive *Acacia* spp. in Europe is relevant in the perspective of finding and evaluating potential natural enemies able to slow the expansion and mitigate the adverse impacts of those species. Since *Acacia* in the broad sense have been grouped into distinct genera, e.g., *Mariosousa*, *Vachellia*, and *Senegalia*, and also other host species in the Leguminosae have been synonymized or renamed, a dedicated study would be required to define the current host range of the bruchid with valid plant names.

Outside its native range, *S. limbatus* has been reported in Hawaii (Bridwell 1920), South America (Oliveira and Costa 2009; Romero Gomez et al. 2009; Meiado et al. 2013), South Africa (Rink 2013), Iran (Boroumand 2010; Ghahari and Borowiec 2017), and United Arab Emirates (Delobel 2011), whereas reports from Mauritius, Saudi Arabia, Yemen, and Oman were unconfirmed (Rink 2013).

In the framework of an international project assessing the risk of invasion of selected alien species (**ALIEM**) (Inghilesi et al. 2018), some *Acacia* spp. seeds were tested in a germination test during which several individuals of *S. limbatus* adults emerged from seed lots of *A. mearnsii* seeds collected in Corsica (France) and Sardinia (Italy) in 2018. This insect species has not been yet recorded in Europe, so that new field collections were planned and carried out in 2019 and 2020.

The main aim of the present study was to investigate the establishment of *S. limbatus* in Sardinia and Corsica according to the traits described by Yus-Ramos et al. (2014) for alien seed beetles, as well as its host association and infestation levels. In addition, a literature search analysis was carried out to provide an updated inventory of host species of *S. limbatus* with valid names, as understanding and predicting host shifts on other *Acacia* species is of pivotal importance in order to define its potential distribution in the Mediterranean Basin.

Materials and methods

Literature search analysis

Data sources used for investigating and updating the host range of *S. limbatus* were retrieved from major online databases, such as Google Scholar, Web of Science, Scopus, CAB abstracts, and ResearchGate. Papers were directly requested to authors and public repositories and libraries whenever inaccessible online. Different combinations of keywords were used in the literature search related to *S. limbatus* and its host range. Whenever possible, references were cross-checked and duplicates removed, giving priority to older records. Original plant names were collected from each reference, whereas country and locality records were reported whenever available.

Plant names were cross-checked taking into account relevant literature and different on-line databases, in particular Seigler et al. (2006), Kyalangalilwa et al. (2013), The Legume Phylogeny Working Group (LPWG 2017), World Flora Online (WFO) (2020), Plants of the World Online (POWO 2020), BHL (for original protologues), and the International Plant Name Index (IPNI) (2020). To our best knowledge, the accepted nomenclature was followed according to current taxonomic standards.

Seed collection

Legumes and loments (hereafter pods) with seeds of *A. mearnsii* were manually collected from adult trees naturalized in Corsica and Sardinia in September-November 2019. Seed sampling was carried out in Sardinia within two Special Areas of Conservation (SACs): "*Berchida e Bidderosa*" (Natura 2000 code ITB020012) (central eastern Sardinia) and "*Monte Linas – Marganai*" (Natura 2000 code ITB041111) (southwestern Sardinia), where the most important populations of *A. mearnsii* are located and the species shows clear invasive traits outcompeting with native vegetation. On the other hand, seeds in Corsica were collected along the eastern side of the island (Fig. 2). In Sardinia, seed sampling was extended to other *Acacia* species, i.e., *Acacia pycnantha* Benth. and *A. saligna*, not previously reported as host species but located nearby the sampling sites of *A. mearnsii*. Following the emergence of *S. limbatus* adults from all *Acacia* species sampled in 2019 (See Results), field collection of seeds was repeated in August-early September 2020 on the same species.

Acacia saligna is a widespread tree species in Corsica and Sardinia (Lozano et al. 2020), in particular along the coast, and severely impacts the characteristics of soils and diversity and structure of the Mediterranean shrublands (Celesti-Grapow et al. 2016; Tozzi et al. 2021). The other two *Acacia* species, although common, are much

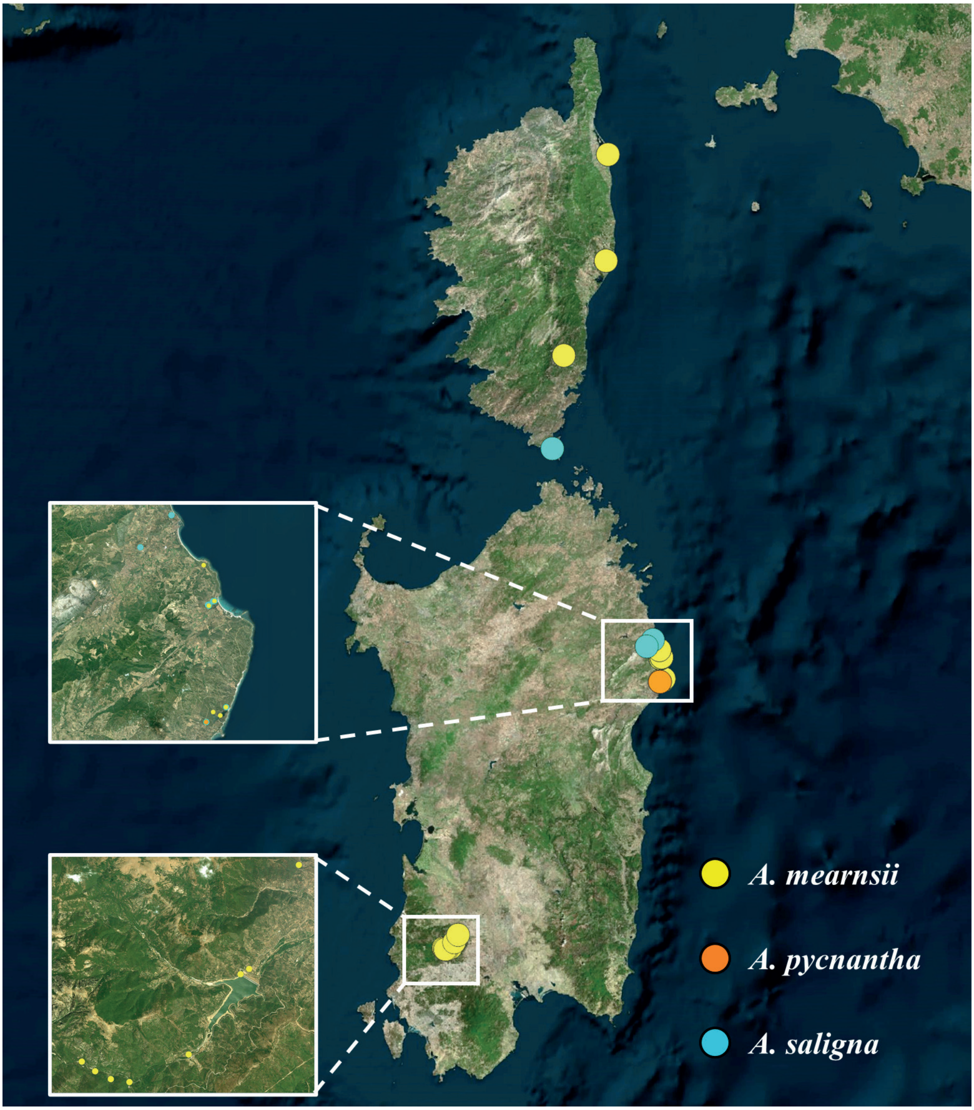

Figure 2. Map of sampling sites of *Acacia* spp. pods and seeds in Sardinia (Italy) and Corsica (France).

less widespread and form dense populations only in a limited number of sites. The width of the sampling site varied widely, ranging from a single tree to tree stands larger than 1,500 m^2, as well as the seed production of trees. Therefore, a minimum of 20 pods per tree, representative of seed production, were collected at random from 1–30 randomly-chosen trees. All in all, the sample size ranged from 75 to 8,500 seeds, depending on the width of the sampling site. In fact, seed production was generally very large in all the investigated *Acacia* spp. in both years and was not a limiting factor in seed sampling.

Seed examination

The collected pods and seeds were stored at laboratory temperature in cardboard envelopes sealed with adhesive tape, to avoid mold development and the escape of tiny seed beetles. Envelopes were opened after approximately three months and beetles were separated and identified morphologically using identification keys for *S. limbatus* adult detection (Johnson 1963; Kingsolver 2004). Seeds were further inspected under a dissecting microscope and the number of *Acacia* spp. seeds with emergence holes was determined in order to calculate the rate of infestation. Seeds of *A. saligna* showed very low seed infestation rates (see Results). However, in view of its importance as an invasive species and in order to point out a potential host shift, the presence of *S. limbatus* eggs on *A. saligna* seeds was also recorded.

Data analysis

The infestation rate, i.e., the percentage of seeds with *S. limbatus* emergence holes, as well as the percentage of *A. saligna* seeds with *S. limbatus* eggs were compared between sites or host species by Fisher exact test. The seed infestation rates were preliminary tested for data overdispersion by analyzing the χ^2 approximation of the residual variance (Venables and Ripley 2002; Zuur et al. 2009). Since overdispersion of data was found, overdispersion parameters were included in the corrected models using a quasibinomial distribution followed by type II ANOVA to test for significance of main effects (Zuur et al. 2009). The seed infestation rate was the response variable, whereas “sampling area” and “year” were the fixed effects in 2019 and 2020, respectively. Corrected analyses were conducted using R software version 4.1.0 (R Development Core Team 2021) at the significance level of 0.05.

Results

Literature search analysis

The literature search on *S. limbatus* host plant species retrieved about 150 references. After a careful nomenclatural revision, the host range of *S. limbatus*, as so far described in literature, includes 37 plant genera belonging to three of the six subfamilies in the family Fabaceae:

subfamily Caesalpinioideae: *Acacia* (16 species), *Acaciella* (2), *Albizia* (10), *Caesalpinia* (1), *Calliandra* (4), *Cassia* (4), *Cercidium* (4), *Chloroleucon* (2), *Delonix* (1), *Desmanthus* (1), *Ebenopsis* (2), *Enterolobium* (2), *Havardia* (4), *Hesperalbizia* (1), *Leucaena* (3), *Lysiloma* (4), *Mariosousa* (4), *Mimosa* (1), *Neptunia* (1), *Painteria* (1), *Parkinsonia* (3), *Piptadenia* (2), *Pithecellobium* (5), *Prosopis* (5), *Pseudopiptadenia* (1), *Pseudosamanea* (1), *Senegalia* (15), *Sphinga* (1), *Vachellia* (2), *Wallaceodendron* (1), and *Zapoteca* (1);

subfamily Cercidoideae: *Bauhinia* (1);
subfamily Papilionoideae: *Arachis* (1), *Butea* (1), *Erythrina* (1), *Glycine* (1), and *Sesbania* (1).

Most host species belong to the subfamily Caesalpinioideae (105), 96 of which to the clade mimosoid, followed by Papilionoideae (5) and a single species of Cercidoideae. The list also comprises the following eight species included as non-host, experimental hosts and uncertain reports: *Calliandra humilis* Benth., *Cercidium texanum* A.Gray, *Delonix regia* (Bojer ex Hook.) Raf., *Prosopis juliflora* (Sw.) DC., *Prosopis velutina* Wooton, *Senegalia ataxacantha* (DC.) Kyal. & Boatwr (syn. *A. ataxacantha* DC.), *Vachellia constricta* (Benth.) Seigler & Ebinger, and *Vachellia farnesiana* (L.) Wight & Arn. (Bridwell 1920; Johnson 1981b; Fox et al. 1996, 2006; Kingsolver 2004; Rink 2013). The comprehensive host range of *S. limbatus* is provided with up-to-date nomenclature of host species on Table 1.

Seed infestation

The field surveys carried out in 2019–2020 demonstrated the presence of the seed-feeding beetle *S. limbatus* both in Sardinia (Italy) and Corsica (France) islands on the host plant *A. mearnsii* (Table 1). In Sardinia, the beetle emerged from seeds collected in all the 14 sites in both the central eastern and southwestern sampling areas. In 2019, the infestation rates ranged from 24.3 to 74.2% and from 39.3 to 83.4% in *Berchida-Bidderosa* and *Monte Linas – Marganai* areas, respectively, showing significant differences among sampling sites (Fisher tests: $\chi^2 = 1074.85$; $df = 5$; $P < 0.001$ and $\chi^2 = 404.83$; $df = 7$; $P < 0.001$, respectively) (Table 1). Overall, the seed infestation rate by *S. limbatus* did not differ between central eastern and southwestern sampling areas ($F = 0.496$; $df = 1.13$; $P = 0.494$). In 2020, the infestation in the central eastern sampling sites also differed significantly among sites (range = 85.4–90.8%) (Fisher test: $\chi^2 = 31.42$; $df = 5$; $P < 0.001$), and increased significantly compared to 2019 ($F = 16.206$; $df = 1.11$; $P = 0.002$). A large majority of *A. mearnsii* seeds (≥ 96.5% of seeds sampled in the various sites) showed *S. limbatus* eggs (up to 18 eggs in a single seed) and ≥ 98.4% of the infested seeds exhibited a single exit hole (Fig. 3A).

Acacia pycnantha trees sampled in central eastern Sardinia in both 2019 and 2020 (site 1) showed the highest infestation levels (85.1 and 95.1%, respectively) compared to *A. mearnsii* sites in the same area (Table 1). Of *A. pycnantha* infested seeds sampled in 2019 and 2020, 29.5 and 45.2%, respectively, exhibited two exit holes and up to 28 eggs were recorded in a single seed (Fig. 3B). Both the percentage of infested seeds and seeds with two holes increased significantly from 2019 to 2020 (Fisher tests: $\chi^2 = 48.73$; $df = 1$; $P < 0.001$ and $\chi^2 = 24.03$; $df = 1$; $P < 0.001$, respectively).

Pods and seeds of *A. saligna* were collected in the surroundings of infested *A. mearnsii* and *A. pycnantha* trees in two and nine sites in central eastern Sardinia (Table 2). The infestation rate was very low in both years and was significantly the highest at the site 5 in both 2019 (4%) (Fisher test: $\chi^2 = 6.32$; $df = 1$; $P = 0.033$) and 2020 (2.6%) (Fisher test: $\chi^2 = 53.74$; $df = 8$; $P < 0.001$). However, *S. limbatus* eggs were recorded on

Table 1. Updated global host range of *Stator limbatus* following a literature search analysis and review of valid plant names.

Host species			Country (Locality)
Host plant valid name †	**Original name in the Reference**	**References**	
Subfamily Caesalpinioideae			
Acacia baileyana F.Muell.	*Acacia baileyana* F. Mueller	Johnson and Kingsolver 1976	USA (California)
Acacia confusa Merr.	*Acacia confusa*	Swezey 1928; Zacher 1952	USA (Hawaii)
Acacia cultriformis A.Cunn. ex G.Don	*Acacia cultriformis* A.Cunn. ex G.Don	Johnson and Kingsolver 1976	
Acacia cyclops A.Cunn. ex G.Don	*Acacia cyclops*	Rink 2013	South Africa (Yzerfontein)
Acacia goldmanii (Britton & Rose) Wiggins	*Acacia goldmanii* (Br. & Rose) Wiggins	Johnson 1979	Mexico
Acacia koa A.Gray	*Acacia koa*	Swezey 1924	USA (Hawaii)
	Acacia koa Gray	Stein 1983	USA (Hawaii)
Acacia leptoclada Benth.	*Acacia leptoclada*	Romero Gomez et al. 2009	
Acacia mangium Willd.	*Acacia mangium* Willd.	Pereira et al. 2004; Medina and Pinzón-Florián 2011; Mojena et al. 2018	Brazil (Mato Grosso, Roraima), Colombia
Acacia mearnsii De Wild.	*Acacia mearnsii* De Wild.	Oliveira and Costa 2009; Cocco et al. (present paper)	Brazil (Rio Grande do Sul), France, Italy
	Acacia mearnsii	Fox et al. 2006; Rink 2013	South Africa (Tokai, Western Cape)
Acacia melanoxylon R.Br.	*Acacia melanoxylon* R.Br.	Johnson and Kingsolver 1976	
Acacia pycnantha Benth.	*Acacia pycnantha* Benth.	Cocco et al. (present paper)	Italy
Acacia podalyriifolia A.Cunn. ex G.Don	*Acacia podalyriifolia* A. Cunningham ex G.Don.	Garlet et al. 2011	Brazil (Rio Grande do Sul)
Acacia retinodes Schltdl.	*Acacia retinodes* Schlect.	Johnson and Kingsolver 1976	USA (California)
Acacia retusa (Jacq.) R.A.Howard	*Acacia retusa* (Jacq.) R.A.Howard	Johnson and Kingsolver 1976	Costa Rica
Acacia richii A.Gray	*Acacia richei* (sic) (*richii*)	Kingsolver 2004	
Acacia saligna (Labill.) H.L.Wendl.	*Acacia saligna* (Labill.) H.L.Wendl.	Cocco et al. (present paper)	Italy, France
Acacia sp.	*Acacia* sp.	Johnson 1984; Boroumand 2010; Ghahari and Borowiec 2017	Guatemala, Iran (Bushehr), Mexico
Acaciella angustissima (Mill.) Britton & Rose	*Acacia angustissima* (Mill.) Kuntze	Johnson and Kingsolver 1976; Johnson 1984, 1995	Colombia, Mexico, USA (Arizona, Texas), Venezuela
	Acacia angustissima	Morse and Farrell 2005a	Mexico, USA (Texas)
	Acacia angustissima angustissima	Kingsolver 2004	
Acaciella goldmanii Britton & Rose	*Acacia macmurphyi* Wiggins	Hetz and Johnson 1988	Mexico
Albizia adinocephala (Donn.Sm.) Britton & Rose ex Record	*Albizzia* (sic) (*Albizia*) *adinocephala*	Janzen 1980	Costa Rica
Albizia berteriana (DC.) Fawc. & Rendle	*Pithecellobium fragrans*	Romero Gomez et al. 2009	
Albizia berteroana (Balb. ex DC.) M.Gómez	*Albizia berteroana*	Romero Gomez et al. 2009	
Albizia caribaea (Urb.) Britton & Rose	*Albizia caribaea* (Urban) Britton & Rose	Johnson 1984	Honduras
	Albizzia (sic) (*Albizia*) *caribaea*	Janzen 1980	Costa Rica
	Albizia caribaea	Romero Gomez et al. 2009	
	Albizia niopoides var. *niopoides*	Romero Gomez et al. 2009	
Albizia chinensis (Osbeck) Merr.	*Albizzia* (sic) (*Albizia*) *chinensis*	Zacher 1952	
Albizia julibrissin Durazz.	*Albizia julibrissin*	Fox et al. 2006	
Albizia lebbeck (L.) Benth.	*Albizia lebbeck* Benth.	Lugo-García et al. 2015	Mexico
	Albizia lebbek (sic) *lebbeck* (L.) Benth.	Hetz and Johnson 1988; Johnson 1995	Mexico, Venezuela
	Albizzia lebbek (sic) (*Albizia lebbeck*)	Bridwell 1920	USA (Hawaii)
	Albizzia (sic) (*Albizia*) *lebbeck* (L.) Benth.	Nascimento 2009	Brazil (Rio de Janeiro)

Host species			**Country (Locality)**
Host plant valid name †	**Original name in the Reference**	**References**	
Albizia saman (Jacq.) Merr.	*Samanea saman*	Bridwell 1920; Morse and Farrell 2005a	Panama, USA (Hawaii), Venezuela
	Pithecolobium (sic) (*Pithecellobium*) (= *Samanea*) *saman*	Zacher 1952	
	Pithecellobium saman (Jacq.) Merrill	Johnson 1984	Guatemala
	Pithecellobium saman (Jacquin) Bentham	Johnson 1995	Ecuador, Venezuela
	Pithecellobium saman	Janzen 1980	Costa Rica
	Samanea saman (Jacq.) Merrill	Johnson and Kingsolver 1976	Costa Rica
Albizia saponaria Blume ex Miq.	*Albizia saponaria*	Kingsolver 2004	
Albizia sinaloensis Britton & Rose	*Albizia sinaloensis* Britt. & Rose	Hetz and Johnson 1988; Johnson 1995	Mexico
Albizia sp.	*Albizia* sp.	Johnson 1984, 1995	Brazil (Rio de Janeiro), Ecuador, Honduras, Venezuela
Caesalpinia pulcherrima (L.) Sw.	*Caesalpinia pulcherrima*	Fox et al. 2006	
Calliandra calothyrsus Meisn.	*Calliandra calothyrsus* Meissn.	Johnson and Lewis 1993	Nicaragua
Calliandra eriophylla Benth.	*Calliandra eriophylla* Bentham	Johnson 1979	USA (Arizona)
Calliandra houstoniana (Mill.) Standl.		Johnson 1984	Mexico, Guatemala
Calliandra houstoniana var. *calothyrsus* (Meissn.) Barneby	*Calliandra confusa* Sprague & Riley	Johnson 1984	Panama
Calliandra humilis Benth. ‡	*Calliandra humilis* ‡	Johnson 1981b	
	Calliandra humilis humilis	Kingsolver 2004	
Calliandra humilis var. *reticulata* (A.Gray) L.D.Benson	*Calliandra humilis reticulata*	Kingsolver 2004	
Calliandra sp.	*Calliandra* sp.	Johnson and Kingsolver 1976; Johnson 1984; Morse and Farrell 2005a	Costa Rica, Mexico, Venezuela
Cassia fistula L.	*Cassia fistula*	Kingsolver 2004	
Cassia grandis L.f.	*Cassia grandis*	Kingsolver 2004	
Cassia javanica L.	*Cassia javanica javanica*	Kingsolver 2004	
Cassia javanica subsp. *nodosa* (Buch.-Ham. ex Roxb.) K.Larsen & S.S.Larsen	*Cassia javanica indochinensis*	Kingsolver 2004	
Cassia moschata Kunth * *Cassia leiandra* Benth. *	*Cassia moschata*	Morse and Farrell 2005b	
Cercidium floridum Torr.	*Cercidium floridum* subsp. *floridum*	Romero Gomez et al. 2009	
	Parkinsonia florida	Kingsolver 2004; Fox et al. 2006	
	Cercidium torreyanum	Zacher 1952	
	Cercidium floridum Bentham	Johnson and Kingsolver 1976	USA (Arizona, California)
	Cercidium floridum (Benth.)	Fox et al. 1996, 2001; Stillwell and Fox 2005	USA (California)
Cercidium macrum I.M.Johnst.	*Parkinsonia texana* var. *macra*	Romero Gomez et al. 2009	
	Parkinsonia texana macra	Kingsolver 2004	
	Parkinsonia macra (Johnst.)	Fox et al. 1996	
	Parkinsonia macra	Nilsson and Johnson 1993	Mexico, USA (Texas)
Cercidium microphyllum Rose & I.M.Johnst.	*Cercidium microphyllum* (Torr.) Rose & Johnst.	Johnson and Kingsolver 1976	Mexico, USA (Arizona)
	Cercidium microphyllum (Benth.)	Fox et al. 2001	USA (California)
	Cercidium microphyllum	Morse and Farrell 2005a	USA (Arizona)
	Parkinsonia microphylla	Stilwell and Fox 2005	
Cercidium texanum A.Gray ‡	*Parkinsonia texana texana*	Kingsolver 2004	
	Parkinsonia texana (A.Gray) S.Watson ‡	Fox et al. 1996	USA (Texas)
Cercidium sp.	*Cercidium* sp.	Johnson 1984	Mexico

Host species			**Country (Locality)**
Host plant valid name †	**Original name in the Reference**	**References**	
Chloroleucon mangense (Jacq.) Britton & Rose	*Chloroleucon mangense*	Morse and Farrell 2005b	
	Chloroleucon mangense (Jacquin) Macbride	Johnson 1995	Venezuela
Chloroleucon tenuiflorum (Benth.) Barneby & J.W.Grimes	*Pithecellobium scalare* Griseb.	Johnson 1984	Brazil (Rio de Janeiro)
Delonix regia (Bojer ex Hook.) Raf. §	*Delonix regia* §	Kingsolver 2004	
Desmanthus bicornutus S.Watson	*Desmanthus bicornutus*	Kingsolver 2004	
Ebenopsis confinis (Standl.) Britton & Rose	*Ebenopsis confinis*	Romero Gomez et al. 2009	
Ebenopsis ebano (Berland.) Barneby & J.W.Grimes	*Ebenopsis ebano*	Romero Gomez et al. 2009	
	Chloroleucon ebano	Nilsson and Johnson 1993	USA (Arizona)
	Pithecellobium ebano	Kingsolver 2004	
	Siderocarpus flexicaule (sic) (*Siderocarpos flexicaulis*)	Cushman 1911	USA (Texas)
Ebenopsis sp.	*Siderocarpus* (sic) (*Siderocarpos*) sp.	Zacher 1952; Romero Gomez et al. 2009	
Enterolobium contortisiliquum (Vell.) Morong	*Enterolobium contortisiliquum* (Vell.) Morong	Meiado et al. 2013	Brazil (Pernambuco)
Enterolobium timbouva Mart.	*Enterolobium timbouva* Mart.	Meiado et al. 2013	Brazil (Pernambuco)
Havardia acatlensis (Benth.) Britton & Rose	*Havardia acatlensis*	Romero Gomez et al. 2009	
Havardia mexicana (Rose) Britton & Rose	*Havardia mexicana*	Romero Gomez et al. 2009	
	Pithecolobium (sic) (*Pithecellobium*) *mexicanum* F. N. Rose	Johnson and Kingsolver 1976	
Havardia pallens (Benth.) Britton & Rose	*Pithecellobium pallens* (Bentham) Standl.	Johnson and Kingsolver 1976	USA (Texas)
	Havardia pallens	Morse and Farrell 2005a	Mexico
	Pithecolobium (sic) (*Pithecellobium*) *brevifolium* Bentham	Johnson and Kingsolver 1976	
Havardia sonorae (S.Watson) Britton & Rose	*Havardia sonorae*	Romero Gomez et al. 2009	
	Pithecellobium sonorae S. Wats.	Johnson and Kingsolver 1976	Mexico
Hesperalbizia occidentalis (Brandegee) Barneby & J.W.Grime	*Albizia plurijuga*	Romero Gomez et al. 2009	Mexico
	Albizia occidentalis Brandegee	Hetz and Johnson 1988	
Leucaena diversifolia (Schltdl.) Benth.	*Leucaena diversifolia*	Romero Gomez et al. 2009	
	Acacia diversifolia	Romero Gomez et al. 2009	
Leucaena leucocephala (Lam.) de Wit	*Leucaena leucocephala* (Lam.) de Wit.	Johnson 1984	Mexico
Leucaena leucocephala subsp. *glabrata* (Rose) Zárate	*Leucaena leucocephala* subsp. *glabrata*	Romero Gomez et al. 2009	
Leucaena pulverulenta (Schltdl.) Benth.	*Leucaena pulverulenta* (Schl.) Bentham	Johnson and Kingsolver 1976	USA (Texas)
Leucaena trichandra (Zucc.) Urb.	*Leucaena diversifolia* subsp. *stenocarpa*	Romero Gomez et al. 2009	
	Leucaena guatemalensis Britt. & Rose	Johnson 1979	Mexico
	Leucaena guatemalensis (Britt. & Rose)	Hetz and Johnson 1988	Mexico
Lysiloma acapulcense (Kunth) Benth.	*Lysiloma acapulcense*	Romero Gomez et al. 2009	Mexico
	Lysiloma acapulcensis (sic) (*acapulcense*) Bentham	Hetz and Johnson 1988	Honduras
	Lysiloma acapulcensis (sic) (*acapulcense*) Kunth. Benth.	Johnson 1984	Guatemala
Lysiloma divaricatum (Jacq.) J.F.Macbr.	*Lysiloma divaricata* (Jacq.) MacBride	Johnson and Kingsolver 1976; Johnson 1984	Mexico
	Lysiloma divaricada (sic) (*divaricata*)	de Lorea Barocio 2006	
	Lysiloma divaricatum	Romero Gomez et al. 2009	
	Lysiloma microphyllum	Romero Gomez et al. 2009	

Host species			**Country (Locality)**
Host plant valid name †	**Original name in the Reference**	**References**	
Lysiloma latisiliquum (L.) Benth.	*Lysiloma latisiliquum* (L.) Benth.	Johnson 1984	Mexico
Lysiloma tergeminum Benth.	*Lysiloma tergeminum*	Romero Gomez et al. 2009	
Lysiloma watsonii Rose	*Lysiloma watsonii*	Romero Gomez et al. 2009	
	Lysiloma thornberi Britt. & Rose	Johnson 1979	USA (Arizona)
	Lysiloma thornberi	Zacher 1952	
	Lysiloma microphylla thornberi	Kingsolver 2004	
	Lysiloma microphyllum var. *thornberi*	Romero Gomez et al. 2009	
Lysiloma sp.	*Lysiloma* sp.	Johnson and Kingsolver 1976; Johnson 1984	Costa Rica; Mexico
Mariosousa acatlensis (Benth.) Seigler & Ebinger	*Acacia acatlensis* Bentham	Johnson and Kingsolver 1976	Mexico
Mariosousa coulteri (Benth.) Seigler & Ebinger	*Acacia coulteri* Bentham	Johnson and Kingsolver 1976	Mexico
	Acacia coulteri	Romero Gomez et al. 2009	
	Mariosousa coulteri	Lugo-Garcia et al. 2015	
	Acacia near *coulteri* Bentham	Johnson and Kingsolver 1976	Mexico
Mariosousa heterophylla (Benth.) Seigler & Ebinger	*Acacia willardiana* Rose	Johnson and Kingsolver 1976	Mexico
Mariosousa millefolia (S.Watson) Seigler & Ebinger	*Acacia millefolia* Wats.	Johnson and Kingsolver 1976	USA (Arizona)
Mimosa distachya var. *laxiflora* (Benth.) Barneby	*Mimosa laxiflora* Benth.	Lugo-García et al. 2015	Mexico
Mimosa sp.	*Mimosa* sp.	de Lorea Barocio 2006; Romero Gomez et al. 2009	Mexico
Neptunia plena (L.) Benth.	*Neptunia plena*	Kingsolver 2004	
Painteria leptophylla (DC.) Britton & Rose	*Painteria leptophylla* (DC.) Britton & Rose	de Jesús Parra-Gil et al. 2020	Mexico
Parkinsonia aculeata L.	*Parkinsonia aculeata* Linnaeus	Johnson and Kingsolver 1976	Mexico, USA (Arizona, Texas)
	Parkinsonia aculeata	Morse and Farrell 2005a	USA (Texas)
	Acacia aculeata	Zacher 1952	
Parkinsonia florida subsp. *peninsulare* (Rose) J.E.Hawkins & Felger	*Cercidium floridum* subsp. *peninsulare*	Romero Gomez et al. 2009	
Parkinsonia praecox (Ruiz & Pav.) Hawkins	*Parkinsonia praecox*	Romero Gomez et al. 2009	
	Cercidium praecox (Ruiz & Pav.) Harms	Johnson and Kingsolver 1976	Mexico
Piptadenia flava (Spreng. ex DC.) Benth.	*Piptadenia flava*	Janzen 1980	Costa Rica
	Parkinsonia flava	Romero Gomez et al. 2009	
Piptadenia obliqua (Pers.) J.F.Macbr.	*Piptadenia obliqua* (Persoon) Macbride	Johnson 1995	Venezuela
	Piptadenia oblique	Morse and Farrell 2005a	Venezuela
Pithecellobium candidum (Kunth) Benth.	*Pithecellobium candidum* Bentham	Johnson 1995	Ecuador
Pithecellobium dulce (Roxb.) Benth.	*Pithecellobium dulce* (Roxb.) Bentham	Johnson and Kingsolver 1976; Johnson 1984, 1995	Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Venezuela
	Pithecellobium dulce	Morse and Farrell 2005a; de Lorea Barocio 2006	Mexico, Ecuador, Venezuela
	Pithecolobium (sic) (*Pithecellobium*) *dulce*	Bridwell 1920; Zacher 1952	USA (Hawaii)
Pithecellobium excelsum (Kunth) Mart.	*Pithecellobium excelsum* Bentham	Johnson 1995	Ecuador
	Pithecellobium excelsum	Morse and Farrell 2005a	Ecuador
Pithecellobium oblongum Benth.	*Pithecellobium oblongum*	Janzen 1980	Costa Rica
Pithecellobium unguis-cati (L.) Benth.	*Pithecellobium unguis-cati*	Morse and Farrell 2005a	Venezuela
	Pithecolobium unguiscatae (sic) (*Pithecellobium unguis-cati*)	Bridwell 1920	USA (California)

Host species			**Country (Locality)**
Host plant valid name †	**Original name in the Reference**	**References**	
Pithecellobium sp.	*Pithecellobium* sp.	Johnson and Kingsolver 1976	El Salvador
	Pithecolobium (sic) (*Pithecellobium*) sp.	Bridwell 1920	USA (Hawaii)
Prosopis chilensis (Molina) Stuntz	*Prosopis chilensis*	Romero Gomez et al. 2009	
	Prosopis chilensis (= *juliflora*)	Zacher 1952	
Prosopis farcta (Banks & Sol.) J.F.Macbr.	*Prosopis farcta*	Boroumand 2010	Iran (Bushehr and Yazd)
	Prosopis farcta (Banks & Soland.) Macbr.	Shamszadeh et al. 2017	Iran (Yazd)
Prosopis glandulosa var *glandulosa* Torr.	*Prosopis glandulosa glandulosa*	Kingsolver 2004	
Prosopis glandulosa var. *torreyana* (L.D.Benson) M.C.Johnst.	*Prosopis glandulosa torreyana*	Kingsolver 2004	
Prosopis juliflora (Sw.) DC. ‡	*Prosopis juliflora* ‡	Bridwell 1920; Kingsolver 2004; Fox et al. 2006	
Prosopis velutina Wooton ‡	*Prosopis velutina* ‡	Johnson 1981b	
Pseudopiptadenia inaequalis (Benth.) Rauschert	*Piptadenia inaequalis* Bentham	Johnson 1995	Venezuela
	Piptadenia inaequalis	Morse and Farrell 2005a	Venezuela
Pseudosamanea guachapele (Kunth) Harms	*Pseudosamanea guachapele*	Amarillo-Suárez et al. 2011	
	Albizia guachepele (sic) (*guachapele*) (HBK.) Dugand	Johnson 1995	Colombia
Senegalia ataxacantha (DC.) Kyal. & Boatwr ‡	*Acacia ataxacantha* ‡	Rink 2013	South Africa
Senegalia berlandieri (Benth.) Britton & Rose	*Acacia berlandieri* Bentham	Johnson and Kingsolver 1976	Mexico, USA (Texas)
	Acacia berlandieri	Amarillo-Suárez et al. 2011	USA (Texas)
Senegalia gaumeri (S.F.Blake) Britton & Rose	*Acacia gaumeri* Blake	Johnson 1984	Honduras, Mexico
	Acacia gaumeri	Morse and Farrell 2005a	Mexico
Senegalia gilliesii (Steud.) Seigler & Ebinger	*Acacia furcatispina*	Romero Gomez et al. 2009	
Senegalia glomerosa (Benth.) Britton & Rose	*Acacia glomerosa*	Romero Gomez et al. 2009	
	Acacia near *glomerosa* Bentham	Johnson and Kingsolver 1976	Mexico
Senegalia greggii (A.Gray) Britton & Rose	*Acacia greggii* A. Gray	Johnson and Kingsolver 1976	Mexico, USA (Arizona, California, Texas)
	Acacia greggii	Morse and Farrell 2005a; Amarillo-Suárez et al. 2011	USA (Arizona)
Senegalia hayesii (Benth.) Britton & Rose	*Acacia hayesii*	Romero Gomez et al. 2009	
Senegalia occidentalis (Rose) Britton & Rose	*Acacia occidentalis* Rose	Johnson and Kingsolver 1976	Mexico
Senegalia picachensis (Brandegee) Britton & Rose	*Acacia picachensis* T. S. Brandg.	Johnson 1984	Mexico
Senegalia polyphylla (DC.) Britton & Rose	*Acacia polyphylla* DC.	Johnson 1995; Johnson and Siemens 1995	Colombia, Venezuela
Senegalia riparia (Kunth) Britton & Rose	*Acacia riparia*	Romero Gomez et al. 2009	
Senegalia roemeriana (Scheele) Britton & Rose	*Acacia roemeriana* Scheele	Johnson and Kingsolver 1976	USA (Texas)
Senegalia tamarindifolia (L.) Britton & Rose	*Acacia tamarindifolia* (L.) Willdenow	Johnson 1995; Johnson and Siemens 1995	Venezuela
	Acacia tamarindifolia	Morse and Farrell 2005a	Martinique
Senegalia tenuifolia (L.) Britton & Rose	*Acacia tenuifolia* (L.) Willd.	Johnson and Kingsolver 1976; Johnson 1984	Costa Rica, Mexico
Senegalia wrightii (Benth.) Britton & Rose	*Acacia wrightii* Bentham	Johnson and Kingsolver 1976	USA (Texas)
	Acacia wrightii	Morse and Farrell 2005a	Mexico, USA (Texas)
Sphinga platyloba (DC.) Barneby & J.W.Grimes	*Sphinga platyloba*	Morse and Farrell 2005b	
	Pithecellobium platyloba (sic) (*platylobum*)	Janzen 1980	Costa Rica
	Havardia platyloba	Romero Gomez et al. 2009	
Vachellia constricta (Benth.) Seigler & Ebinger ‡	*Acacia constricta* ‡	Johnson 1981b	
Vachellia farnesiana (L.) Wight & Arn. ‡	*Acacia farnesiana* ‡	Bridwell 1920	
	Acacia farnesiana	Zacher 1952	

Host species			Country (Locality)
Host plant valid name †	**Original name in the Reference**	**References**	
Wallaceodendron celebicum Koord.	*Wallaceodendron celebicum*	Bryan 1932	USA (Hawaii)
Zapoteca portoricensis (Jacq.) H.M.Hern.	*Zapoteca portoricensis*	Morse and Farrell 2005b	
Subfamily Cercidoideae			
Bauhinia purpurea L.	*Bauhinia purpurea* L.	Fox et al. 2006	
Subfamily Papilionoideae			
Arachis hypogaea L.	*Arachis hypogaea*	Kingsolver 2004	
Butea monosperma (Lam.) Kunze	*Butea monosperma*	Romero Gomez et al. 2009	
	Erythrina monosperma	Zacher 1952	
Erythrina sandwicensis O.Deg.	*Erythrina sandwicensis*	Kingsolver 2004	
Glycine max (L.) Merr.	*Glycine max*	Kingsolver 2004	
Sesbania sp.	*Sesbania* sp.	Romero Gomez et al. 2009	

† Valid names following Kyalangalilwa et al. (2013), Plants of the World Online (POWO 2020), and World Flora Online (WFO) (2020). ‡ Non-host or experimental hosts. * Morse and Farrell (2005b) did not specify the authorship, it is therefore impossible to determine whether they referred to *Cassia moschata* Kunth or *Cassia leiandra* Benth, which are both accepted names. § Uncertain report (Kingsolver 2004).

up to 52.8 and 79.6% of *A. saligna* seeds in 2019 and 2020, respectively (Fig. 3C). A single seed harbored up to six eggs. The seed infestation rate ranged in 2020 from 0 to 2.6% regardless of the distance from infested *Acacia* spp. trees, whereas *A. saligna* seeds with the highest percentage of beetle eggs (sites 1, 4, 5, and 6, range 45.1–79.6%) were recorded on trees <5 m apart from infested trees (Table 2).

In Corsica, *S. limbatus* adults were recorded in all four sampling sites. In 2019, adults emerged in both eastern (site 19) and northeastern (site 18) sites from *A. mearnsii* seeds. Most seeds exhibited exit holes and egg chorions of *S. limbatus*, although a few individuals were recorded: four adults from site 19 and one from site 18. In 2020, *S. limbatus* adults were further recovered in sites 18 and 21, in which more than 400

Figure 3. *Acacia* seeds (with arils on top) infested by *Stator limbatus*, with eggs and exit holes **A** *S. limbatus* adult emerging from an *Acacia mearnsii* seed with 11 eggs **B** *S. limbatus* adult emerging from *A. pycnantha* seed with two exit holes **C** *A. saligna* seed with a *S. limbatus* egg and one exit hole.

Table 2. Locations of sampling sites in Sardinia (Italy) and Corsica (France), and seed infestation rates of *Acacia pycnantha* and *A. mearnsii* by *Stator limbatus*.

Site no.	WGS84 Coordinates (°N, °E)	Sampling date	Host plant	Sampled seeds (no.)	Infestation rate (%) †
Sardinia, *Berchida-Bidderosa* area, 2019					
1	40.451995, 9.778190	18/09/2019	*A. pycnantha*	315	85.1 a
2	40.459980, 9.785646	18/09/2019	*A. mearnsii*	199	38.7 d
3	40.457190, 9.793082	18/09/2019, 01/10/2019	*A. mearnsii*	3459	74.2 b
4	40.463992, 9.798704	18/09/2019, 01/10/2019	*A. mearnsii*	1030	49.3 d
5	40.545390, 9.782090	18/09/2019	*A. mearnsii*	61	45.9 d
6	40.549220, 9.788000	18/09/2019, 01/10/2019	*A. mearnsii*	1137	24.3 e
7	40.578073, 9.777057	18/09/2019, 01/10/2019	*A. mearnsii*	3639	67.5 c
Sardinia, *Berchida-Bidderosa* area, 2020					
1	40.451995, 9.778190	10/08/2020	*A. pycnantha*	2415	95.1 a
2	40.459980, 9.785646	10/08/2020	*A. mearnsii*	1784	90.8 b
3	40.457190, 9.793082	10/08/2020	*A. mearnsii*	2234	89.0 bc
4	40.463992, 9.798704	10/08/2020	*A. mearnsii*	1704	86.5 d
5	40.545390, 9.782090	10/08/2020	*A. mearnsii*	1023	85.4 d
6	40.578073, 9.777057	10/08/2020	*A. mearnsii*	390	87.2 cd
7	40.549220, 9.788000	10/08/2020	*A. mearnsii*	1574	89.8 bc
Sardinia, *Monte Linas – Marganai* area, 2019					
10	39.421480, 8.716520	23/09/2019	*A. mearnsii*	226	61.9 cde
11	39.398540, 8.695790	23/09/2019	*A. mearnsii*	199	54.3 e
12	39.391094, 8.675427	23/09/2019	*A. mearnsii*	341	65.4 cd
13	39.396532, 8.658998	23/09/2019	*A. mearnsii*	671	66.6 c
14	39.393961, 8.663604	23/09/2019	*A. mearnsii*	980	59.8 de
15	39.391863, 8.669016	23/09/2019	*A. mearnsii*	951	79.4 b
16	39.420067, 8.713574	23/09/2019	*A. mearnsii*	1187	83.4 a
17	39.449340, 8.733530	23/09/2019	*A. mearnsii*	428	39.3 f
Corsica, 2019					
18	42.546699, 9.525582	29/10/2019	*A. mearnsii*	-	n.a.
19	42.125300, 9.510656	07/11/2019	*A. mearnsii*	-	n.a.
Corsica, 2020					
18	42.546576, 9.5246522	20/08/2020	*A. mearnsii*	-	n.a.
19	42.125065, 9.510606	20/08/2020	*A. mearnsii*	8500	56.0
21	41.380217, 9.222299	03/09/2020	*A. mearnsii*	-	n.a.

† Different letters within years indicate significant difference by Fisher exact test ($P < 0.05$). n.a. = not available.

Table 3. Locations of sampling sites in Sardinia (Italy) and Corsica (France), and seed infestation rates of *Acacia saligna* seeds by *Stator limbatus*.

Site no.	WGS84 Coordinates (°N, °E)	Sampling date	Distance from infested *Acacia* trees	Sampled seeds (no.)	Infestation rate (%) †	Seeds with *S. limbatus* eggs (%) †
Sardinia, *Berchida-Bidderosa* area, 2019						
4	40.463799, 9.799295	18/09/2019	< 5 m	156	0 b	44.9 a
5	40.545420, 9.782050	18/09/2019	< 5 m	75	4.0 a	52.8 a
Sardinia, *Berchida-Bidderosa* area, 2020						
1	40.451980, 9.778390	10/08/2020	< 5 m	1550	0 d	57.2 b
4	40.463799, 9.799295	10/08/2020	< 5 m	524	0.6 abc	60.7 b
5	40.545420, 9.782050	10/08/2020	< 5 m	116	2.6 a	79.6 a
	40.546396, 9.782224	10/08/2020	< 100 m	864	0.3 bcd	24.4 d
	40.546109, 9.781190	10/08/2020	< 100 m	867	0 d	18.0 e
6	40.549240, 9.788131	10/08/2020	< 5 m	859	0 d	45.1 c
	40.549022, 9.786670	10/08/2020	< 100 m	1237	0.2 bcd	22.5 d
8	40.618420, 9.743740	10/08/2020	> 100 m	981	0 d	3.0 g
9	40.592818, 9.710812	17/08/2020	> 100 m	596	0.2 bcd	8.9 f
Corsica, 2020						
20	41.380217, 9.222299	27/08/2020	-	4360	0.2	n.a.

† Different letters within years indicate significant difference by Fisher exact test ($P < 0.05$).

adults emerged from samples of *A. mearnsii* seeds of unknown sizes. In site 19, the infestation level by *S. limbatus* was 56.0%. Seeds of *A. saligna* were collected in site 20, where the infestation rate was 0.2%.

Discussion

The extensive collection of *S. limbatus* during the field surveys in 2019 and 2020 in Sardinia and Corsica following the first record in 2018 indicates that the seed beetle has found suitable climatic conditions and has established in Europe. *Stator limbatus* can be considered established according to the definition of Yus-Ramos et al. (2014), i.e., a species able to reproduce successfully in natural ecosystems. *Stator limbatus* exhibits biological features that could support its further spread in Europe. At first, this species has a wide host range worldwide, with about 15 species reported in Europe (Euro+Med 2021; GBIF 2021). Furthermore, its native geographic range includes diverse climates, spanning from dry forests of northern South America to deserts of Central America and southwestern United States (Stillwell and Fox 2009). In addition, this bruchid developed under laboratory conditions also on non-native species, including *Acacia cyclops* A.Cunn. G.Don and *S. ataxacantha* (syn. *A. ataxacantha*) (native to Australia and South Africa, respectively) (Rink 2013), as well as non-host species, such as *C. humilis*, *C. texanum*, *P. juliflora*, *P. velutina*, *V. constricta*, and *V. farnesiana* (Bridwell 1920; Johnson 1981b; Fox et al. 1996). Finally, *S. limbatus* have shown adaptive oviposition phenotypic plasticity in response to host species, as fewer and bigger eggs are laid on exotic or unfavorable hosts (Amarillo-Suarez et al. 2017). Such maternal egg-size plasticity is suggested to be an ancestral trait influencing the evolution of the diet breadth (Amarillo-Suárez and Fox 2006). Overall, the wide presence of host species of *S. limbatus* in Europe, its strong host shift potential, and climate adaptation suggest its possible spread in Mediterranean environments, and its presence in unsampled areas cannot be ruled out.

This species was recovered from *Acacia* spp. seeds in Sardinia, in multiple sites distant up to 150 km, and Corsica, in four areas distant about 130 km. Even though the country of first introduction in Europe remains undetermined, the wide presence of this alien insect in distant areas supports the hypothesis that its introduction occurred several years ago. The introduction of *S. limbatus* in Europe was most likely accidental and its detection unexpected. The pathway of first introduction is presently unknown, as no specific custom interception has so far been reported. With regard to pathways of secondary spread, in view of its wide host range and endophytic behavior of larvae, we may assume that it was introduced through movement of contaminated commodities, i.e., plants for planting, as a parasite of seeds (CBD 2014; Faulkner et al. 2020). In fact, after its first introduction, a secondary spread pathway may have occurred as a result of movement of contaminated plants (with pods) or seeds of *A. saligna*, *A. mearnsii*, and *A. pycnantha*, which are commonly planted in southern Europe and significantly traded. In addition, the very large number of different host species should be taken into account (Table 1), as many are common ornamental, i.e., *Albizia* spp., *Leucaena* spp., *Parkinsonia* spp., and *Glycine max* (L.) Merr., or forestry and multipurpose species, i.e., *Acacia* spp., in the Mediterranean area. Therefore, in order to investigate the *S. limbatus* presence or intercept its introduction in areas nearby Sardinia and Corsica, specific monitoring plans on its host species should be set up in southern France and mainland Italy. Although the pathways of first introduction and secondary spread are generally not known for bruchid seed beetles, several authors suggest introductions through importation of seed or nursery stocks of host plant species for ornamental or forestry purposes, e.g., *Bruchidius terrenus* (Sharp, 1886) on *Albizia julibrissin* Durazz. and *Amblycerus robiniae* (Fabricius, 1781) on *Gleditsia triacanthos* L. in the United States (Kingsolver 2004; Hoebeke et al. 2009; Yus-Ramos et al. 2014).

The introduction of alien seed beetles in Europe shows an increasing trend in the last 20 years, in accordance with the worldwide trend described by Seebens et al. (2017). Beenen and Roques (2010) reported 14 Bruchinae alien species in Europe, seven of which introduced before 1900, three species in the period 1901–1950, two in 1951–2000, and finally two species reported from 2001 to 2010. Yus-Ramos et al. (2014) further extended the list of alien bruchids in Europe to a total of 42 species, including four recent introductions, namely *Bruchidius radiannae* Anton & Delobel, 2003 and *Caryedon acaciae* (Gyllenhal, 1833) on *Vachellia karroo* (Hayne) Banfi & Galasso (syn. *Acacia karroo* Hayne) in 2007 in Spain (Yus Ramos and Coello García 2007, 2008), *Acanthoscelides macrophthalmus* (Schaeffer, 1907) on *Leucaena leucocephala* (Lam.) de Wit in Cyprus in 2007 (Vassiliou and Papadoulis 2008), and *B. terrenus* on *A. julibrissin* in Bulgaria in 2009 (Stojanova 2010). Furthermore, *A. robiniae* was reported on *G. triacanthos* in Romania in 2018 following an unconfirmed report in Hungary in 1986 (Rădac et al. 2021). Therefore, according to literature reports, seven species of bruchids have been reported in Europe in the last 20 years. In both Corsica and Sardinia, *S. limbatus* larvae developed on seeds of *A. mearnsii*, a tree native to Australia which has shown to be invasive in Europe, South America, and Africa. This insect-host association has been previously reported in Brazil, where an infestation rate of 44.3% was observed (Oliveira and Costa 2009), and South Africa (Rink 2013). *Acacia mearnsii* is cultivated in Brazil for tannins, cellulose, and charcoal production (Garlet et al. 2011), whereas in Europe and in South Africa, presently, this species has a lower significant economic importance and is rather invasive (Souza-Alonso et al. 2017; Railoun et al. 2021).

In Sardinia, beetle adults emerged abundantly also from *A. pycnantha* seeds, and, interestingly, 45% of sampled seeds showed two exit holes, differently from *A. mearnsii* seeds which showed a single exit hole. This brings evidence that *A. pycnantha* seeds support the development of more than one larva of *S. limbatus*, most likely because of the bigger size of its seeds compared to those of *A. mearnsii*. In central eastern Sardinia, the infestation rate was more homogeneous among sampling sites in 2020 than in 2019, as the range decreased from 49.9% (24.3–74.2%) in 2019 to 5.4% (85.4–90.8%) in 2020. Moreover, infestation rates increased significantly on both *A. mearnsii* and *A. pycnantha*. However, the seed production of trees in the sampling sites was not quantitatively estimated being beyond the aims of the study. Estimates of seed infestation rates with no assessment of tree seed production and over such a short period, i.e. two years, prevent to infer on spatio-temporal population trends of *S. limbatus*. The same insect abundance can, in fact, cause high infestation rates in the event of low seed production or low rates when seed production is high. Nonetheless, although *Acacia* spp. seed production and accumulation may vary widely, Australian and African species usually produce large or very large quantities of seed and may have large soil-stored seed banks (Gibson et al. 2011). High production of seeds for the three investigated species has been observed both in the native and in the invaded ranges, e.g., *A. mearnsii* in South Africa (Impson et al. 2021), being one of the drivers of invasiveness at the global level. Indeed, large amounts of pods were observed on *Acacia* spp. trees as well as seeds in the topsoil in both 2019 and 2020 (A. Cocco, Y. Petit, pers. obs.). Furthermore, high numbers of seedlings were observed in the sampling sites with *A. mearnsii*.

Previous studies on infestation by *S. limbatus* on Fabaceae species reported seed damages of 15% on *E. timbouva* (Meiado et al. 2013), 19% on *Acacia mangium* Willd. (Mojena et al. 2018), and 70% on *Acacia podalyriifolia* A.Cunn. ex G.Don (Garlet et al. 2011) in Brazil. In Mexico, seed infestation rates of 16.8% were observed on *Painteria leptophylla* (DC.) Britton & Rose (de Jesús Parra-Gil et al. 2020) and 33.6% on *Mariosousa coulteri* (Benth.) Seigler & Ebinger by both *S. limbatus* and *Merobruchus santarosae* Kingsolver, 1989 (Coleoptera, Chrysomelidae) (Romero Gomez et al. 2009). Susceptibility to *S. limbatus* widely varied among hosts and areas; however, comparisons are difficult, as seed infestation rates are influenced by a number of abiotic and biotic factors, including seed availability and environmental conditions. Despite its recent report in South Africa, *S. limbatus* has not been reported infesting *A. pycnantha* seeds (Rink 2013; Magona et al. 2018).

A word of caution is in order with regard to *A. saligna* as a host species for *S. limbatus*. In fact, infestation rates were very low in both years and countries, and the highest values (4% in 2019 and 2.6% 2020) were observed in the same site. Nonetheless, infestation by *S. limbatus* on *A. saligna* seeds was not limited to a single site, as infested plants were observed in both Sardinia and Corsica. Moreover, beetle eggs were observed on up to 80% of *A. saligna* seeds, especially on plants near to infested *Acacia* spp. trees. This could be due to an opportunistic egg-laying behavior on the nearest alternative hosts. Furthermore, oviposition on *A. saligna* indicates that seeds had no antixenotic effect on female oviposition and oviposition is promoted by suitable hosts nearby. Chemical or physical barriers on *A. saligna* seeds preventing larval development cannot be ruled out and would require further investigations. Laboratory tests carried out in South Africa investigating the oviposition preference showed that *S. limbatus* females accepted *A. saligna* seeds for oviposition, together with seeds of *A. cyclops*, *A. mearnsii*, *Paraserianthes lophantha* (Willd.) I.C.Nielsen (invasive non-native species in South Africa), and *Vachellia tortilis* (Forssk.) Galasso & Banfi [syn. *Acacia tortilis* (Forssk.) Hayne], *S. ataxacantha*, *Senegalia caffra* (Thunb.) P.J.Hurter & Mabb. [syn. *A. caffra* (Thunb.) Willd.], *Senegalia nigrescens* (Oliv.) P.J.Hurter [syn. *A. nigrescens* (Oliv.)] and *Vachellia sieberiana* var. *woodii* (Burtt Davy) Kyal. & Boatwr. [syn. *A.*

sieberiana var. *woodii* (Burtt Davy) Keay & Brenan] (native species to South Africa). However, adults emerged only from *A. mearnsii*, *A. cyclops*, and *S. ataxacantha*, indicating that food availability may not be the only factor limiting the larval development (Rink 2013).

In view of its high seed infestation rates, *S. limbatus* has been suggested to play a role as biocontrol agent of invasive non-native *Acacia* species (Rink 2013). In South Africa, extensive biological control programs have been developed against invasive tree species, as, for example, the release of *A. macrophthalmus* for biological control of *L. leucocephala* in 1999 (Olckers 2004). Five seed-weevil *Melanterius* spp. (Coleoptera, Curculionidae) were introduced from Australia in different periods to reduce the invasiveness of *P. lophantha* and ten *Acacia* spp., including the three species investigated in the present paper, i.e., *A. mearnsii*, *A. saligna*, and *A. pycnantha* (Impson et al. 2011). Seed damage caused by weevils varied largely among sites and years from 4% to over 90%. Such variability was explained by a specific 4-year study on *Melanterius*–*Acacia* relationship and was mostly due to variable seed quality that resulted in low larval and pupal survival rates (Impson and Hoffmann 2019). Overall, seed-feeders are unlikely to effectively reduce the *Acacia* spp. density as a stand-alone control agent due to the extraordinarily high prolificacy of plants resulting in huge accumulation of long-lived seeds in the soil. In fact, effective results were obtained through the release of the flower-galling midge, *Dasineura rubiformis* Kolesik (Diptera, Cecidomyiidae) complemented by a seed-feeding weevil, *Melanterius maculatus* Lea (Coleoptera, Curculionidae), which caused a strong reduction of seed production of *A. mearnsii* (Impson et al. 2021). This reduction is expected to curb the accumulation rate of the seed banks and, in the medium-long term, reduce the spread of the invasive species. Besides a potential biocontrol agent of invasive plant species, further beneficial environmental effects by *S. limbatus* may be represented by the promotion of seed germination, e.g., on *Enterolobium contortisiliquum* (Vell.) Morong and *E. timbouva* Mart. (Meiado et al. 2013).

The present findings indicate the adaptability of *S. limbatus* to new host species when established in new areas. *Stator limbatus* also showed phenotypic plasticity in response to seed size or seed quality (Amarillo-Suárez and Fox 2006), in accordance with findings in other species (Hardy et al. 1992; Tsai et al. 2001). Moreover, this is consistent with results from studies showing that development time decreased and adult mass increased when insects developed on high quality hosts (Lindroth et al. 1991; Stockhoff 1993). Therefore, host shifts on local plants and new host associations cannot be ruled out in Europe in view of its ability to accept and adapt to local hosts. Adaptation to new or non-preferred host species has been observed on other coleopteran alien species, such as the red palm weevil *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera, Dryophthoridae) on the dwarf palm, *Chamaerops humilis* L. (Cocco et al. 2019). Importantly, *S. limbatus* has been reported on > 90 host species and ≥ 20 genera (de Jesús Parra-Gil et al. 2020), which is one of the widest host ranges within the Bruchinae. In view of its tropic spectrum, it has been classified as polyphagous, i.e., feeding in the seeds of various plant genera of different subfamilies (Ribeiro-Costa and

Almeida 2012; Yus-Ramos 2018). However, its host use is widely variable and it shows local specialization depending on the diversity of available host species (Morse and Farrell 2005a, 2005b). The establishment of *S. limbatus* in Europe and new associations with *A. pycnantha* and *A. saligna* required a redefinition and update of the bruchid host range to facilitate further research on its potential adaptation and spread in Europe. The exact definition of the host range of *S. limbatus* is not trivial due to nomenclatural issues within the family Fabaceae which have not been resolved (LPWG 2017). In addition, in a number of cases, the literature reported incorrect or partial names for the host plants. The bibliographic search analysis allowed to extend the global host range of *S. limbatus* to 111 species, in most part belonging to the mimosoid clade of the subfamily Caesalpinioideae (Fabaceae) (LPWG 2017). Synonym issues were resolved, e.g., *Acacia diversifolia* and *Leucaena diversifolia* both mentioned by Romero Gomez et al (2009) and synonymized in *Leucaena diversifolia* (Schltdl.) Benth, and up-to-date nomenclature provide the current and comprehensive overview of the feeding spectrum of *S. limbatus*. However, some old or unconfirmed reports would require further investigations, e.g., *G. max*, *Wallaceodendron celebicum* Koord., and *Arachis hypogaea* L. (Brian 1932; Kingsolver 2004). Since no previous records were found in literature, *A. pycnantha* and *A. saligna* are included in the present paper for the first time in the host range of *S. limbatus*.

This report of establishment of *S. limbatus* in Europe contributes to updating the insect worldwide distribution, which now includes North and Central America (native region), South America, South Africa, the Middle East, and southern Europe. Future research is required on known and potential host species in order to investigate its potential distribution and new host associations with native or non-native plant species (Parry et al. 2013). Studies on suitable climatic conditions for *S. limbatus* development will further assess the risks of spread in the Mediterranean Basin. Such surveys should include also urban habitats, in which seed feeders are frequently found (Branco et al. 2019).

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Seed viability of common ragweed (*Ambrosia artemisiifolia* L.) is affected by seed origin and age, but also by testing method and laboratory

Rea Maria Hall[1,2], Bernhard Urban[1,2], Hana Skálová[3], Lenka Moravcová[3], Ulrike Sölter[4], Uwe Starfinger[5], Gabriela Kazinczi[6], Johan van Valkenburg[7], Annamaria Fenesi[8], Bojan Konstantinovic[9], Ahmet Uludag[10], Suzanne Lommen[11,12], Gerhard Karrer[1]

1 *University of Natural Resources and Life Science, Vienna, Department of Integrative Biology and Biodiversity Research, Institute of Botany; Gregor Mendel Strasse 33, 1180 Vienna, Austria* **2** *University of Natural Resources and Life Science, Vienna, Department of Crop Science, Institute of Agronomy; Konrad Lorenz Straße 24, 3430 Tulln an der Donau, Austria* **3** *Czech Academy of Science, Department of Invasion Ecology, Institute of Botany; Zámek 1, 25243 Průhonice, Czech Republic* **4** *Julius Kuehn-Institut; Institute for National and International Plant Health; Messeweg 11/12, 38104 Braunschweig, Germany* **5** *Julius Kuehn-Institut; Institute for National and International Plant Health; Messeweg 11/12, 38104 Braunschweig, Germany* **6** *Hungarian University of Agriculture and Life Sciences, Institute of Plant Protection, Department of Plant Protection, Deák F. str. 16, 8360 Keszthely, Hungary* **7** *National Reference Centre for Plant Health, Netherlands, Food and Consumer Product Safety Authority; Geertjesweg 15, 6706 EA Wageningen, The Netherlands* **8** *Babeş-Bolyai University, Hungarian Department of Biology and Ecology, Republicii Street 42, RO-400015 Cluj-Napoca, Romania* **9** *University of Novi Sad, Faculty of Agriculture, Department of Environmental and Plant Protection; Trg Dositeja Obradovica 8, 21000 Novi Sad, Serbia* **10** *Çanakkale Onsekiz Mart Üniversitesi, Ziraat Fakültesi; Cumhuriyet Mah. Kepez, 17100, Çanakkale, Turkey* **11** *Leiden University, Institute of Biology, 2333BE, Leiden, The Netherlands* **12** *Université de Fribourg, Department of Biology, Champs du Musée 10, 1700 Fribourg, Switzerland*

Corresponding author: Rea Maria Hall (rea.hall@boku.ac.at)

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Abstract

Common ragweed (*Ambrosia artemisiifolia* L.) is an annual *Asteraceae* species native to North America which is highly invasive across Europe and has harmful impacts, especially on human health and agriculture. Besides its wide ecological range, particularly its high reproductive power by seeds is promoting its spread to various habitats and regions. To prevent further spread and to control the plant, the European Commission funded projects and COST-Actions involving scientists from all over Europe. A joint trial was set up comprising eight different laboratories from Europe to study seed viability variation in different seed samples. Three different testing methods (viability test with 2,3,5-triphenyltetrazolium chloride (TTC), a germination test combined with a subsequent TTC test and a crush test) were tested within the EU-COST-Action SMARTER network to four different seed origins. The viability test results from different laboratories were compared for variation amongst tests and laboratories. The main aim was to optimise the reliability of testing procedures, but results revealed not only significant effects of seed origin and seed age on seed viability, but also considerable differences between the output of the individual testing methods and furthermore between laboratories.

Due to these significant differences in the results of the testing labs, additionally a second test was set up. Twelve Austrian ragweed populations were used for TTC testing to obtain a precise adjustment of the testing method as well as a tight guideline for interpreting the results, particularly for the TTC state "intermediate" since a proper classification of TTC-intermediate coloured seeds is still a challenge when determining viability rates.

Keywords
Common ragweed, germination rate, seed age, seed origin, 2,3,5-triphenyltetrazolium chloride (TTC), viability testing, crush test

Introduction

Information on seed viability is of significant importance, not only in agricultural science, but also in the field of invasion ecology. Beyond fecundity and dispersal capacities, the fate of seeds of invasive alien species (IAS) after arrival to a new site is essential in determining the outcome of invasion (Moravcová et al. 2006; Fumanal et al. 2008). Especially for annual invasive alien species, production and performance of seeds is the main driver of naturalisation (Richardson et al. 2000). In this context, seed persistence is often associated with invasion success, since the ability of delaying seed germination through time is a bet-hedging strategy that spreads the risk of reproductive failure. This is essential in unpredictable, newly-conquered environments where the risk of dying before reaching maturity is high (Venable and Brown 1988; Ooi 2012; Gioria et al. 2012; Long et al. 2015). Thus, most of the IAS are ruderals that are well adapted to disturbances due to their long-lived seed banks (Grime 2001; Fumanal et al. 2008). One of the economically most important IAS in Europe is common ragweed (*Ambrosia artemisiifolia* L.), an annual *Asteraceae* species native to North America which is not only one of the most dominant inducers of pollen allergy, but also a troublesome agronomic weed (Fumanal et al. 2007; Bullock 2010; Smith et al. 2013; Schaffner et al. 2020). Extensive populations of common ragweed are known in Austria, Croatia, France, Germany Hungary, Italy, Romania, Russia, Serbia, Slovenia, Switzerland, (Northern) Turkey and Ukraine (Kazinczi et al. 2008; Essl et al. 2015; Ozaslan et al. 2016; Zambak and Uludağ 2019). Its large ecological amplitude enables the species to be a successful pioneer in early successional stages and in several habitat types (Fumanal et al. 2008a). In agricultural areas, common ragweed is one of the most important weeds in spring crops like sunflower, soybean, maize, sugar beet and oil seed pumpkin. Additionally, it also colonises other human-disturbed habitats, such as roadsides, construction sites, abandoned gravel pits and riverbanks (Fumanal et al. 2008b; Essl et al. 2015,). Last, but not least, common ragweed is a serious threat to human health due to abundant allergenic pollen release. The pollen production varies amongst plants and years from 0.1 to 3.8 billion pollen grains per plant which become airborne immediately when conditions are favourable (Fumanal et al. 2007; Smith et al. 2013; Katz and Batterman 2019). In North America and parts of Europe, the pollen of the species is the main cause of hay fever and allergic rhinitis, causing an estimated financial burden for the health system of approx. 630 Euro per year per each person concerned (Wopfner et al. 2005; Jäger 2006; Ziska et al. 2011). Schaffner et al. (2020) even estimated direct and indirect costs caused by common ragweed in the European Union by Euro 7.4 billion per year.

Particularly, its success as an IAS is tightly associated with its high production of seeds. On average, one plant produces 1,500 to 3,000 seeds. The highest reported number of seeds per plant has been found in Russia with a total number of approx. 62,000 seeds on one single plant (Fisjunov 1984). The dispersal unit of common ragweed is often called "achene". It consists of a durable involucrum covering a hard-coated fruit (= achene s. str); the single seed (morphological term!) inside the achene is soft and comprises of a well-developed embryo. For simplicity, the term "seed" will be used in the subsequent text to describe the dispersal unit of common ragweed. When seeds of common ragweed mature in autumn, they are innately dormant (primary dormancy). Seeds in primary dormancy require moist chilling (cold stratification) to come out of dormancy, which occurs during winter (Payne and Kleinschmidt 1961; Baskin and Baskin 1980). Under laboratory conditions, Baskin and Baskin (1987) recommended a wet and dark stratification at 4 °C for 2 weeks to obtain about 75% of germination. Since Pickett and Baskin (1973) demonstrated higher germination rates with increasing length of stratification, chilling treatments of 6 weeks and more are recommended for maximum germination percentage by other authors (Willemsen and Rice 1972; Leiblein et al. 2014; Onen et al. 2020). However, less is known about storage suitability of common ragweed seeds. In general, the life span of seeds is determined by their genetic and physiological storage potential and by any deteriorating events that occur prior to or during storage, as well as by the interaction with environmental factors (Bewley and Black 1994). Even though Toole and Brown (1946) stated that seeds of common ragweed can remain dormant in the soil seed bank for up to 39 years, it is evident that long-term storage of seeds generally reduces their viability and vigour, even if the seeds are stored *ex situ* under stable conditions (Kazinczi et al. 2008; Long et al. 2015; Starfinger and Karrer 2016).

Seed viability and performance is crucial to understand the ecological niche and expansion of annual weeds, i.e. when weed management systems are to be established (Zimdahl 2018). Seed viability is commonly evaluated by a germination test, colouration test with 2,3,5-triphenyltetrazolium chloride (TTC) and crush test. It is not clear at the present time which test gives most reliable results and which is most easily applicable.

To prevent further spread and to control or eradicate this IAS, the European Commission funded the project "Assessing and controlling the spread and the effects of common ragweed in Europe". Within the framework of this project ("HALT AMBROSIA"), a consortium of scientists from five countries established a viability test by colouration of living ragweed seeds by TTC. The first results indicated that differences in TTC classification of different seed lots by different labs were higher than the variation between the seed origins (Starfinger et al. 2012; Karrer et al. 2016b). Hitherto existing results about seed biology were transferred to many stakeholders via the EU-funded FA 1203 COST-action SMARTER (Sustainable management of *Ambrosia artemisiifolia* in Europe, Müller-Schärer et al. 2018), an interdisciplinary network of more than 120 experts involved in the control of common ragweed in more than 30 countries.

The main goal of this paper is to achieve better insight into germination biology and viability testing as part of monitoring tools against ragweed. Therefore, in 2015 a joint trial was set up within SMARTER, comprising eight different European laboratories (including the five labs from the first joint trial within HALT AMBROSIA), to evaluate three different viability testing methods (colouration of living tissue by TTC, germination test combined with a subsequent TTC test and a crush test). We used four different seed origins (two different sites each, in Austria and in Hungary), aiming at the optimisation of testing procedures on the viability of ragweed seeds. The specific aim of this study was to detect possible differences in the viability status between: 1) the seed origins and ages, 2) the testing labs and 3) the testing methods.

Due to disagreements of the participating labs on the classification of the TTC-stained seeds, particularly concerning the TTC-state "intermediate", in a second step the experimental set-up of the joint trial was extended by further germination/TTC test and stand-alone TTC test to: 4) obtain a precise adjustment of the testing method, as well as a tight guideline for interpreting the results, particularly for the TTC state "intermediate" because a proper classification of these seeds is still a challenge when determining viability rates.

Material and methods

Joint trial (test comparison by the SMARTER team)

Plant material

Mature, dry seeds of common ragweed were collected from the years 2011 to 2014 on four different sites in Hungary and Austria (Table 1).

Immediately after collection, seeds were dried at room temperature, air purified and placed at 4 °C ± 2 °C in a dark refrigeration chamber until the beginning of the experiment. Eight institutions participated in the joint trial which started in 2015 (Table 2).

Table 1. Locations, year of collection, coordinates, habitat type and 100 kernel weight of the two Hungarian and two Austrian seed origins of common ragweed analysed in the joint trial.

Population	Year of collection	Coordinates	Habitat type	100 kernel weight (mean ± std)
Hungary 1 (H1-2011) Kaposvar	2011	46°22'07.70"N, 17°51'07.90"E	arable field	3.621 ± 1.128
Austria 1 (A1-2012) Hagenbrunn	2012	48°19'56.90"N, 16°24'21.77"E	ruderal area	5.424 ± 1.642
Austria 2 (A2-2013) Seyring	2013	48°19'55.96"N, 16°29'15.04"E	ruderal meadow	4.778 ± 2.065
Hungary 2 (H2-2014) Kaposvar	2014	46°22'06.30"N, 17°50'59.50"E	arable field	3.565 ± 1.292

Table 2. Participating institutions in the joint trial.

Institution	Country	Number of testers
University of Natural Resources and Life Science Vienna (AT)	Austria	2
Czech Academy of Science, Pruhonice (CZ)	Czech Republic	1
Julius Kuehn-Institut Braunschweig (D)	Germany	1
Kaposvár University (H)	Hungary	1
NL Food and Consumer Product Safety Authority (NL)	Netherlands	2
Babeş-Bolyai University, Cluj (RO)	Romania	2
University of Novi Sad (SRB)	Serbia	1
Düzce University (TR)	Turkey	1

2,3,5-triphenyltetrazolium chloride (TTC) test procedure

The TTC assay is a fast evaluation for seed viability. Respiring tissues are capable of converting a colourless compound to a carmine-red coloured water-insoluble formazan by hydrogen transfer reaction, catalysed by the cellular dehydrogenase. TTC enters both living and dead cells, but only living cells catalyse the formazan, resulting in colouration of these tissues (Moussa et al. 2013).

For the first run of TTC testing, 50 intact seeds from each locality (Austria 1 + 2 and Hungary 1 + 2) were selected and soaked with tap water for 12–15 hours. After soaking, the seeds were cut with a medical scalpel longitudinally into two halves and the presence of the embryo was checked using a microscope. Seeds with obviously intact embryos were placed in 0.5 ml PCR-tubes that were filled with 1%-TTC in demineralised water and were incubated for 24 hours at 30 °C in darkness. Afterwards, the embryos of the seeds were checked under a microscope to determine if there was a discolouration according to the TTC-staining-protocol provided by COST ACTION FA1203 (following Starfinger and Karrer 2016), which determines the three different categories "alive", "intermediate" and "dead" (Fig. 1, Table 3). According to this protocol, completely discoloured seeds are classified as fully viable, but behaving as dormant, completely non-coloured seeds are classified as dead, since there are no living cells, which would have changed colour when treated with TTC and partial discoloured seeds are seen to be in an intermediate stage. The same procedure with again 50 seeds from each locality was repeated in a second run, which was executed by another independent observer. In case of the labs AT, NL and RO, the staining of all tested seeds was evaluated twice, namely by two different observers independently.

In the joint trial, it became obvious that especially the number of non-viable classified seeds per seed lot varied heavily between labs due to the circumstance that these

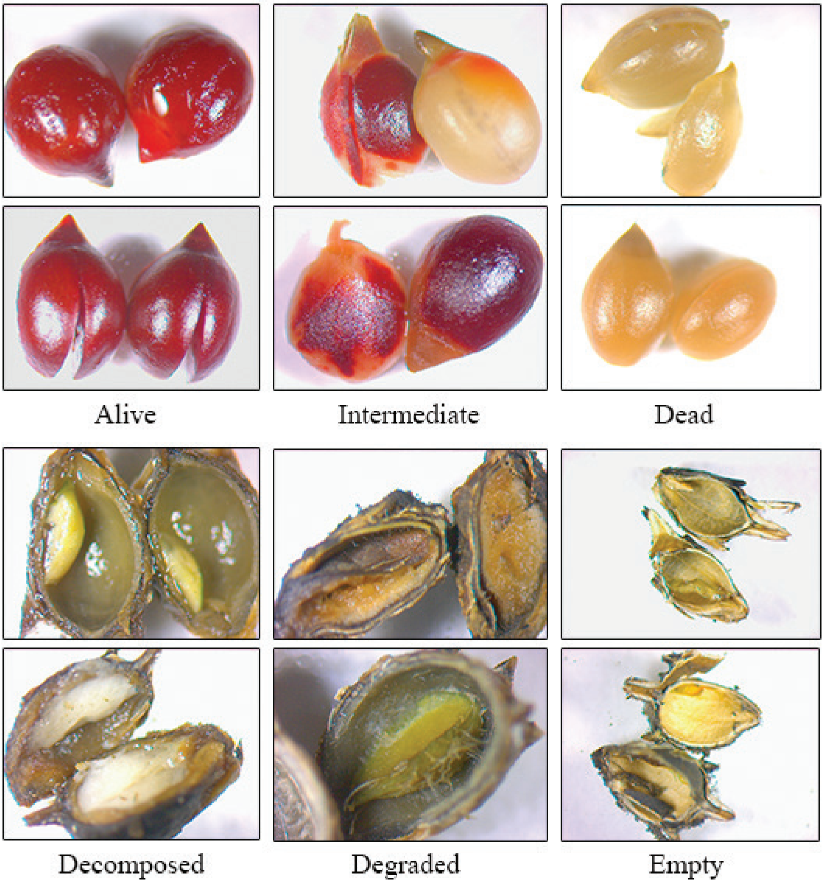

Figure 1. The six classification categories of seeds in the TTC test.

Table 3. Description of the possible TTC states in TTC test.

Viability status	Description
positive "alive"	both halves of the embryo are completely, deep carmine-red-coloured
intermediate	partial discolouration on the halves of the embryo
negative "dead"	both halves of the embryo show no discolouration
decomposed	all seeds in which the embryo showed severe decomposition
degraded	seed which had dried out and had already started to decay
empty	seeds in which the embryo did not develop or had completely decayed

seeds were not only obviously dead (no discolouration in TTC) or empty (Karrer et al. 2016b). Thus, for the extended trial, two more state categories (decomposed, degraded) were introduced to avoid bias in the results (Fig. 1, Table 3). Those decomposed or degraded seeds had an embryo and also showed discolouration, but these embryos had deformations, showed indications for drying-out-effects or liquefaction. Thus, the seeds with decomposed or degraded embryos were calculated in the sum of TTC-negative tested seeds.

Germination test

In a first run, the test was performed on 50 seeds from each of the seed lots Austria 1 and Austria 2, as well as of Hungary 1 and Hungary 2. Stratification of the first set of seed origins was done in Petri dishes with 9 cm diameter, which were filled with fine quartz sand (0.1–0.7 mm grain size), building a layer of approximately 0.5 cm thickness. After placing the unsterilised seeds (17, 17, 16 seeds per dish) on the surface of this quartz sand layer, 10 ml tap water was added, Petri dishes were closed with the upper shell and seeds were stratified at 4 °C for six weeks in darkness. This procedure was repeated approximately two weeks after the first run with a second set of 50 seeds from each locality, respectively.

After six weeks of stratification, the Petri dishes were moved to a climate chamber with 12 hours full light at 25 °C and 12 hours darkness at 15 °C (optimum conditions for ragweed germination defined by Leiblein-Wild et al. 2014 and Farooq et al. 2019) and incubated for 28 days. Petri dishes were checked three times per week. Seeds with a visible radicle were recorded as germinated and removed (Karrer et al. 2016c). Seeds which did not germinate within the 28-days-period were afterwards tested with TTC following the same protocol as mentioned above, except for soaking, due to the fact that seeds were continuously kept wet during the germination test. Fully coloured TTC-tested seeds were counted as viable together with the germinated seeds.

Crush test

From each location and year, 100 intact seeds were cut longitudinally into two halves. The larger half seed was placed on a filter paper on a glass slide with the cut side touching the paper. Each seed half was crushed by placing another glass slide on top and pressing firmly all the way down to the paper. When the seeds caused liquid staining after crushing on the filter paper, the seed was considered alive. If the filter paper was dry or the external intact seed was empty, the seed was considered dead (Karrer et al. 2016c).

Extended trial

The extension of viability tests was executed in the BOKU lab only. In the extended trial, mature dry seeds of common ragweed were randomly selected from twelve populations, which were harvested from the years 2010 to 2014 from ten plants growing on twelve different sites in south-eastern parts of Austria (Table 4). Immediately after collection, seeds were dried at room temperature, air purified and placed at 4 °C in a refrigeration chamber until the beginning of the experiment. Germination tests plus subsequent TTC tests, as well as a stand-alone TTC test, were performed on parallel subsamples of the seed lots following the same protocol as with the joint trial. For each test, 300 obviously intact seeds of each of the twelve populations were randomly selected. A total of 100 of them were weighed and measured for length and width.

Table 4. Locations, year of collection, coordinates and habitat type of the 12 Austrian seed origins of common ragweed analysed in the extended-trial.

Location	Pop. code	Year of collection	Coordinates	Habitat type
Seyring	SEY	2014	48°19'55.96"N, 16°29'15.04"E	ruderal site
Hartberg	HAR	2013	47°16'53.44"N, 15°58'22.91"E	roadside
Fürstenfeld	FÜF	2013	47°2'53.55"N, 16°4'48.76"E	roadside
Halbenrain	HAL	2013	46°43'20.95"N, 15°56'50.93"E	arable field
Neunkirchen	NEK	2013	47°43'33.96"N, 16°4'52.26"E	arable field
Sankt Pölten	STP	2013	48°12'12.96"N, 15°38'18.44"E	roadside
Zillingtal	ZIL	2012	47°47'12.93"N, 16°26'47.61"E	arable field
Leobendorf	LEO	2012	48°22'31.92"N, 16°19'32.75"E	arable field
Neue Donau	NDO	2012	48°12'59.68"N, 16°25'45.84"E	ruderal site
Deutsch Wagram	DWA	2010	48°17'56.59"N, 16°33'50.44"E	roadside
Unterpurkla	UPU	2010	46°43'54.48"N, 15°54'11.30"E	arable field
Hagenbrunn	HAG	2010	48°19'56.90"N, 16°24'21.77"E	ruderal site

Furthermore, various studies showed that the carbon/nitrogen-ratio (C/N-ratio) has a severe impact on the seed viability and their ability to germinate. For example, medium levels of maternal nitrogen (N) led to medium N-levels in the offspring, which subsequently accelerated germination. Additionally, it was observed that nitrate provided by the mother plant acts as a signal molecule to seed dormancy breakage (Holdsworth et al. 2008; Karimmojeni et al. 2014). Thus, the average nitrogen (N) and carbon (C) concentration and subsequently the C/N-ratio of 50 randomly selected seeds per population was determined by the Dumas Combustion Method (Winkler et al. 2000), using an elemental analyser (vario MAX cube CNS, Elementar Analysesysteme GmbH, Germany). This procedure was replicated for all seed origins 10 times (n = 5,000 seeds per population).

Data analysis

Germination rate

The final germination rate (germinated versus non-germinated seeds) was used as a primary dependent variable for analysis.

Mean germination time

The mean germination time (MGT) is a dimensionless indicator of the germination performance, opposing the germination rate and the temporal distribution of germination of each single seed and is calculated according to Ellis and Roberts (1980) as follows:

$$\mathrm{MGT} = \Sigma\,(t \times n)\,/\,\Sigma\,n,$$

t – time in days; n – is the number of seeds which completed germination on day t. Lower MGT values indicate faster germination.

Statistical analysis

Sigma Plot 12.5 was used for graphical visualisation of the results. Statistical analyses were performed using software SAS version 9.2. Analysis of variance (PROC GLM) was used to test the influence of the independent factors origin, age and testing lab on germination rate and/or seed viability. Subsequently, multiple comparisons of means according to Student-Newman Keuls were performed. Means were separated by least significant differences (LSD), when the F-test indicated factorial effects on the significance level of $p < 0.05$. The Shapiro Wilk test was used to test the normal distribution of data and Levene's test was used to check equality of variances. If normal distribution were not given, a Kruskal-Wallis ANOVA on Ranks was performed. If homogeneity of variances were not given, statistical analysis was executed, using Welsh's test of unequal variances t-test.

Logistic regression analyses (PROC LOGISTIC) was performed to evaluate the significance of the explanatory factor origin and sampling year. Due to sufficient replications, particularly in the extended joint trail, we further tested if there is significant influence of the habitat type on the results. Linear regressions models (PROC REG) were used to test the influence of all the factors on seed viability and on the different viability states gained with TTC testing.

In the extended trial, randomized samples for the germination test and subsequent TTC test, as well as for the stand-alone TTC test, were drawn from twelve different populations. One prerequisite when testing the viability of seeds with different testing methods is homogeneity of samples. To check if the randomised samples for germination test and subsequent TTC test, as well as for the stand-alone TTC test, are comparable, a Chi^2-test was performed. Since only embryo-bearing seeds can be viable (able to germinate or TTC-stained), the parameter "intact embryo" was used as the indicator to evaluate the probability of samples deriving from same population.

Probability of an intermediate stained seed to be viable

TTC-positive and TTC-negative seeds are quite easy to determine, but the intermediate state covers a wide range of different colouration intensities. Since germination tests with TTC-treated seeds are not possible, a statistical analysis (Chi^2-test) on the basis of the results of germination test and TTC test was performed to calculate the probability of a TTC-intermediate tested seed being viable or not.

Results

Joint trial

Germination rate

Germination was tested by all eight labs participating in the joint trail. The Turkish lab (TR) only reported results on the germination rate. Information on the mean germination time was not submitted.

The origin and the sampling year had a significant impact (age; F = 19.89; p < 0.001) on the germination rate of common ragweed seeds (Fig. 2). Interaction effects of these two factors were not significant (F = 0.316; p = 0.579). In all laboratories participating within the joint trial, H1-2014 showed the highest germination rate which accounted for 74.7% on average, followed by the population A2-2013 with a mean of 69.4%. These two younger seed lots differed significantly from the older seed lots A1-2012 and H2-2011, the latter accounting for an average germination rate of 36.5% and 25.4%, respectively (Table 5). Even though the results varied widely between labs, the factor lab had no significant influence (ANOVA: F = 0.948; p = 0.483) on the germination test results (Table 5).

Mean Germination Time (MGT)

The year of harvest (age) had a significant impact on the MGT of the seeds of common ragweed (F = 174.76; p < 0.001, Table 5, Suppl. material 1: Fig. S1). The fastest germination activity of 0.118 on average was observed with the seeds of A2-2013, deriving from a ruderal meadow north of Vienna. A total of 50% of the seeds of this population germinated within the first eight days after incubation. In contrast, the seeds from A1-2012 took the longest incubation time before germination with an MGT of 0.449 (median: 11 days after trial start). Contrary to the germination rate, the MGT varied widely amongst labs, even though all participants followed the same

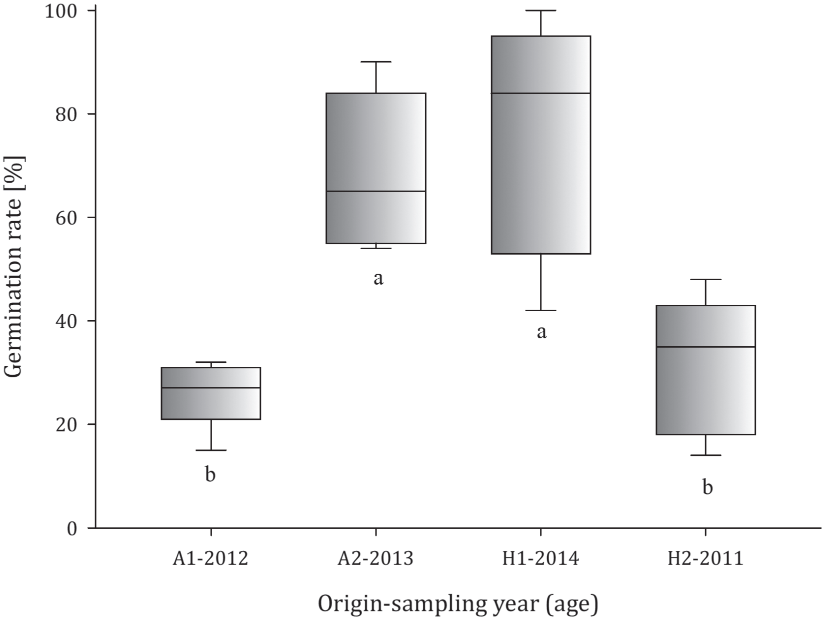

Figure 2. Germination rate [%] of common ragweed seeds in dependency of the factors origin and year (n = 700/population/year ; Two-way-ANOVA, different letters indicate significant differences).

Table 5. Results of the eight independent laboratories for the germination rate [%], mean germination time, share [%] of ragweed seeds tested alive, intermediate and dead in stand-alone TTC test, as well as the share [%] of common ragweed seeds tested alive with crush-test, with regard to the four different origins of the seeds (A1-2012, A2-2013, H1-2014, H2-2011; two numbers in the columns show the result of two individual testers).

	Austria (AT)	**Czech Rep. (CZ)**	**Germany (D)**	**Hungary (H)**	**Nether-lands (NL)**	**Romania (RO)**	**Serbia (SRB)**	**Turkey* (TR)**	**overall mean**
A1-2012									
Germination test									
Germination rate [%]	31	21	15	27	29	23	32	40	**25.4**
MGT	0.251	0.370	0.822	0.209	0.314	0.518	0.660	–	**0.449**
Stand-alone TTC test									
positive [%]	19 | 21	3	29	6	27 | 24	29 | 36	12	8	**19.5**
intermediate [%]	25 | 20	21	8	20	6 | 9	50 | 45	0	12	**19.6**
dead [%]	56 | 59	76	63	74	67 | 67	21 | 19	88	80	**60.9**
Crush-test: alive [%]	94	97	100	70	98	98	78	100	**91.9**
A2-2013									
Germination test									
Germination rate [%]	64	74	65	55	54	90	84	74	**69.4**
MGT	0.072	0.065	0.154	0.087	0.133	0.121	0.197	–	**0.118**
Stand-alone TTC test									
positive [%]	54 | 47	46	75.0	26.0	76 | 78	61 | 67	60	23	**55.7**
intermediate [%]	26 | 30	37	8.0	40.0	6 | 4	28 | 21	0	20	**20.0**
dead [%]	20 | 23	17	17.0	34.0	18 | 18	11 | 12	40	57	**24.3**
Crush-test: alive [%]	89	99	95	54	94	99	100	97	**90.9**
H1-2014									
Germination test									
Germination rate [%]	84	91	53	42	58	95	100	76	**74.7**
MGT	0.087	0.115	0.301	0.132	0.192	0.157	0.172	–	**0.165**
Stand-alone TTC test									
positive [%]	76 | 84	74	86	36	90 | 94	85 | 92	86	64	**78.8**
intermediate [%]	22 | 14	15	0	28	7 | 2	12 | 5	2	21	**11.6**
dead [%]	2 | 2	11	14	36	3 | 4	3 | 3	12	15	**9.6**
Crush-test: alive [%]	97	82	91	66	95	96	96	100	**90.4**
H2-2011									
Germination test									
Germination rate [%]	28	35	14	43	18	48	70	36	**36.5**
MGT	0.281	0.260	0.969	0.099	0.623	0.336	0.243	–	**0.402**
Stand-alone TTC test									
positive [%]	36 | 37	29	62	20	43 | 66	56 | 62	54	29	**44.9**
intermediate [%]	33 | 30	51	26	28	28 | 9	37 | 33	0	13	**26.2**
dead [%]	31 | 33	20	12	52	29 | 25	7 | 5	46	58	**28.9**
Crush-test: alive [%]	84	88	97	56	93	98	98	96	**88.8**

* the Turkish lab only reported the results for the germination rate; information on the mean germination time was not available.

incubation protocol. However, statistical analysis showed that the various labs had significantly different results (F = 22.4; $p < 0.001$; Fig. 3). Except for A2-2013, all other seeds which were tested in the German lab showed a significantly higher MGT than all other laboratories. On average, in this lab, it took 14 days until 50% of the ragweed seeds germinated, whereas in the Hungarian lab, it took only 4 days until half of the seeds germinated. Similar results were observed with seeds tested in the Romanian, the Dutch and the Serbian laboratory, respectively, which all showed significantly higher MGT than all other labs, particularly with seed lots A2-2013, H1-2014 and H2-2011.

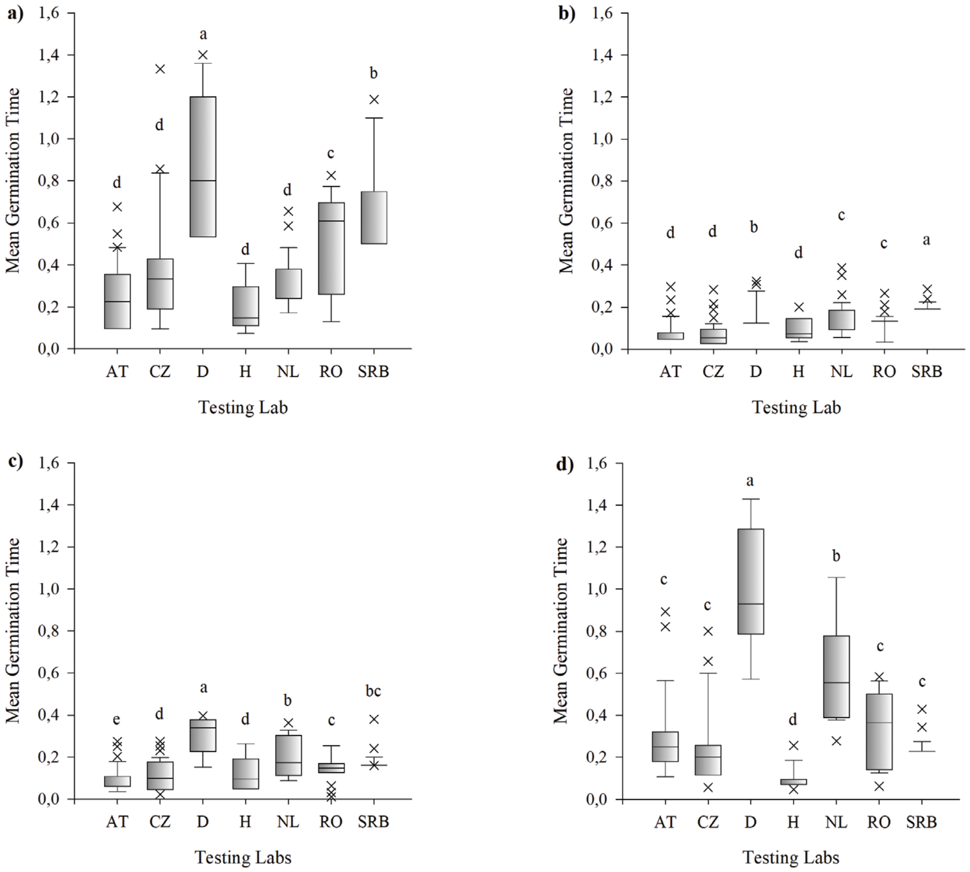

Figure 3. Mean germination time of ragweed seeds from populations **a** A1-2012 **b** A2-2013 **c** H1-2014 and **d** H2-2011 in relation to the factor testing lab (n = 700 seeds/population; 100 seeds/lab; different letters indicate significant differences); for country codes, see Table 5.

Stand-alone TTC test

As with germination, the factors age and origin (F = 28.36; $p < 0.001$), respectively, had significant impact on the results of the stand-alone TTC test within the joint trial (Table 5, Suppl. material 1: Fig. S2). On average, H1-2014 had a share of 78.8% TTC-positive tested seeds. A total of 9.6% of these seeds were classified as TTC-negative and 11.6% were classified as intermediate. The seeds A2-2013 showed on average, over all laboratories, a share of 55.7% viable seeds and 20.0% were classified as intermediate. Even though H2-2011 were the oldest from all samples, the share of TTC-positive tested seeds accounted for 44.9% and was, therefore, significantly higher than with the seeds of A1-2012 which had the significantly lowest share of TTC-positive tested seeds (19.5%), as well as the significantly highest share of TTC-negative tested seeds (60.9%).

Variation amongst labs

Significant differences in TTC-test results were also observed amongst labs (Fig. 4). Especially with the seed lots A2-2013, H1-2014 and H2-2011, the Hungarian lab had the significantly lowest share of viable seeds, if viable seeds consist of TTC-positives only, as well as in the case of intermediates being included into the group of viable seeds. The highest average number of viable (= TTC-positive tested) seeds (65.5%) was observed by tester 2 of the Dutch lab, but this did not differ significantly neither from the results of the first Dutch tester nor from the results of various other labs. Particularly with the TTC-state "intermediate", the Serbian lab observed the significantly lowest share of intermediate seeds with 0.5%. Especially with populations A1-2012, A2-2013 and H2-2011, not a single seed was classified as intermediate with the Serbian lab. The highest share of intermediate stained seeds of 31.8% was measured by Romanian tester

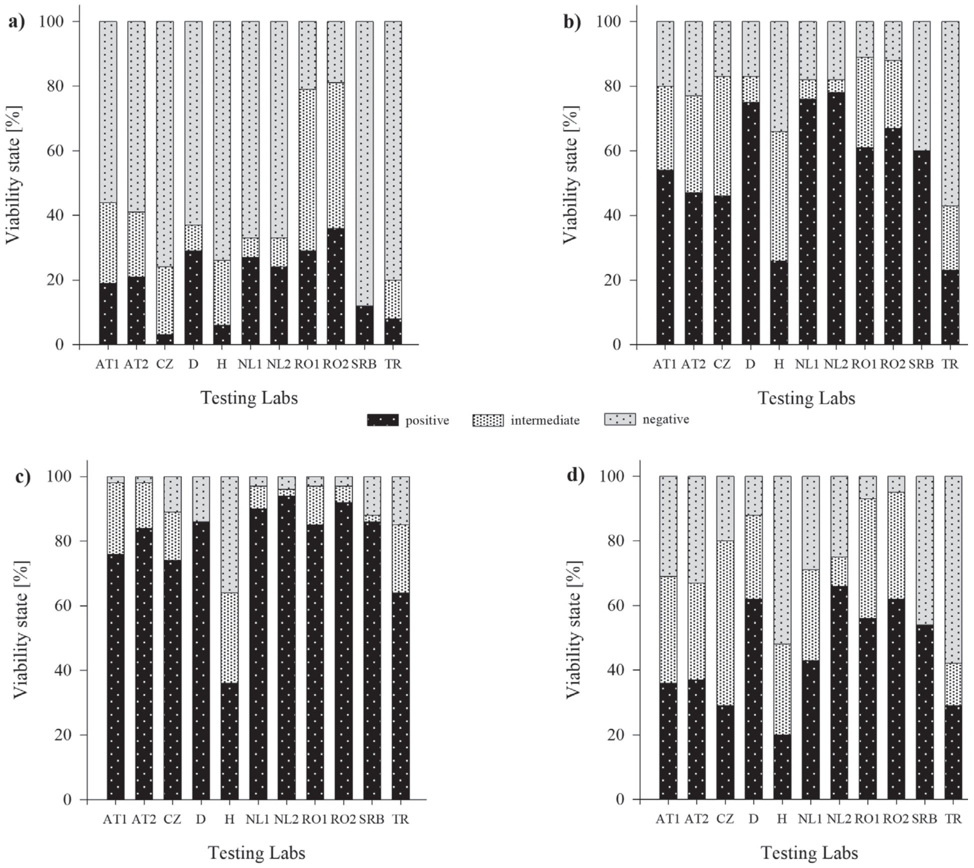

Figure 4. Relative frequency [%] of the viability states of ragweed seeds in the stand-alone TTC test depending on the testing laboratory for **a** population A1-2012 **b** A2-2013 **c** H1-2014 and **d** population H2-2011. In Austria (AT1 and AT2), The Netherlands (NL1 and NL2) and Romania (RO1 and RO2), seeds were tested by two individual testers (n = 4,400).

1, but this result was not significantly higher than some other labs. In contrast, testers Romania 1 and Romania 2 had the significantly lowest share of TTC-negative classified seeds within their samples (Suppl. material 1: Table S1).

Crush-test

As shown in Table 5 and Fig. 5, the crush test led to a significant overestimation of viability of ragweed seeds in five out of eight labs, irrespective of the year of sampling (age) and/or origin of seeds. Only in the Hungarian lab, the number of viable seeds tested with the crush test was comparable with the results of the germination test. On a lab level, the highest discrepancy amongst the germination test, TTC test and crush test was observed in the Turkish lab, where the crush test showed an overestimation of 55.6% on average compared to both the other testing methods. With respect to the populations, the highest average discrepancy in results was observed with ragweed population A1-2012: On average, 25.4% of the seeds germinated and 19.5% were tested positive in the stand-alone TTC test, but with the crush test, a viability rate of 91.9% was computed.

The Extended Trial

Table 6 summarises the results of germination test and subsequent TTC test, as well as stand-alone TTC test confirming the results of the joint trial as we found significant impact of the factors origin and age (year of sampling) on the germination rate (Fig. 6 and Suppl. material 1: Fig. S3), viability rate and mean germination time of ragweed seeds.

The highest mean germination rate of 96.0% was observed with seeds from Seyring (SEY) which had also the shortest MGT of 0.05, whereas the lowest germination rate of 3.0% on average was shown by the seeds deriving from Hagenbrunn (HAG) which showed the highest MGT of 1.33 (Table 6; Fig. 6b; Suppl. material 1: Fig. S4).

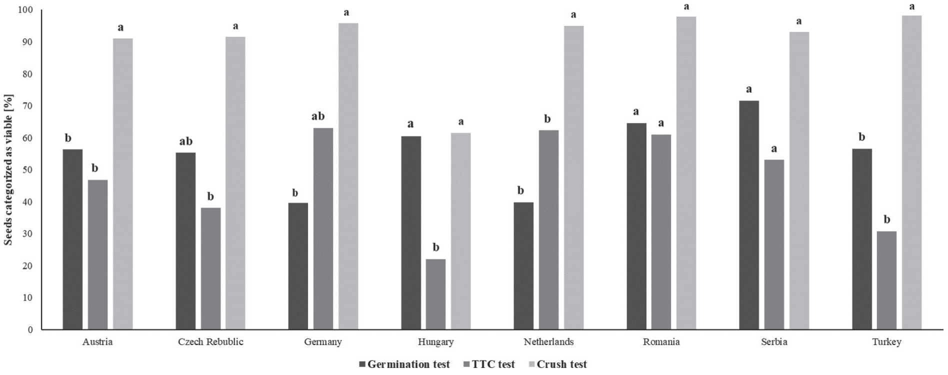

Figure 5. Percentage of seeds tested as viable with the germination test, stand-alone TTC test and crushtest on average over four populations depending on the factor testing lab (n = 300 per population and testing lab; different letters indicate significant differences).

Table 6. Germination rate [%] and the share of seeds [%] tested as viable, intermediate or non-viable in either germination test plus subsequent TTC test or stand-alone TTC test, the mean germination time (2-tailed t-test, n = 7,200; superscript letters indicate significant differences between the respective values from different seed origin or from different sampling years, respectively).

Origin	**Germination test + subsequent TTC test**				**Stand-alone TTC test**			Mean germination time
	Germination rate [%]	TTC-positive [%]	TTC-intermediate [%]	TTC-negative* [%]	TTC-positive [%]	TTC-intermediate [%]	TTC-negative* [%]	
Seyring (SEY)	96.0 ^{a}	1.5	0.0	2.5	87.0	1.5	11.5	0.046 ^{a}
Hartberg (HAR)	74.0 ^{b}	4.0	3.5	18.5	66.0	15.0	19.0	0.072 ^{ab}
Fürstenfeld (FUF)	63.5 ^{c}	9.5	5.0	22.0	40.0	37.0	23.0	0.096 ^{ab}
Halbenrain (HAL)	75.0 ^{b}	7.0	6.5	11.5	68.0	13.5	18.5	0.066 ^{ab}
Neunkirchen (NEK)	53.5 ^{c}	14.0	12.0	20.5	48.5	23.5	28.0	0.151 ^{abc}
Sankt Pölten (STP)	36.0 ^{de}	12.0	25.0	27.0	26.0	42.5	31.5	0.216 ^{c}
Zillingtal (ZIL)	22.5 ^{f}	16.0	29.0	32.5	6.0	57.0	37.0	0.418 ^{d}
Leobendorf (LEO)	44.0 ^{d}	10.0	21.5	24.5	19.0	38.0	43.0	0.175 ^{bc}
Neue Donau (NDO)	25.0 ^{ef}	10.0	41.0	24.0	22.5	36.5	41.0	0.253 ^{c}
Dt. Wagram (DWA)	16.5 ^{f}	8.5	20.5	54.5	11.5	56.5	32.0	0.487 ^{d}
Unterpurkla (UPU)	7.0 ^{g}	1.0	30.0	62.0	2.5	29.5	68.0	0.796 ^{e}
Hagenbrunn (HAG)	3.0 ^{g}	1.5	33.5	62.0	0.0	34.0	66.0	1.333 ^{f}
Year								
2014	96.0 ^{a}	1.5	0.0	2.5	87	1.5	11.5	0.046 ^{a}
2013	60.4 ^{b}	9.3	10.4	19.9	49.7	26.3	24.0	0.120 ^{b}
2012	30.5 ^{c}	12.0	30.5	27.0	15.8	43.8	40.3	0.282 ^{c}
2010	8.8 ^{d}	3.7	28.0	59.5	4.7	40.0	50.3	0.872 ^{d}

*Seeds with degraded or decomposed embryo were calculated in the sum of TTC-negative tested seeds.

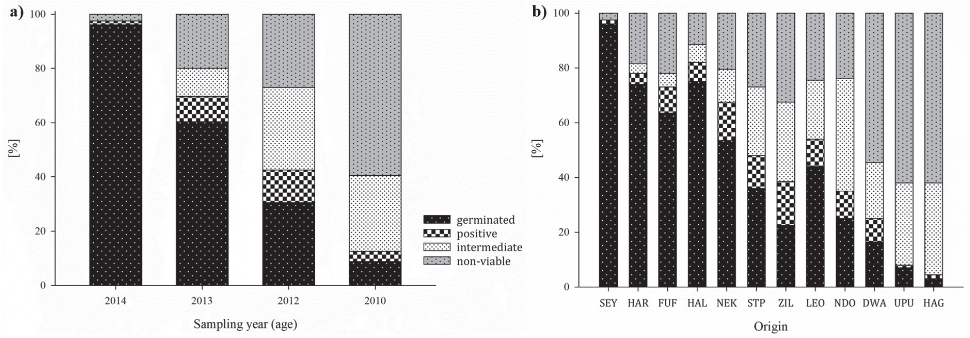

Figure 6. Germination rate [%; black-dotted bars) of ragweed seeds from 12 Austrian origins, as well as the share of positive, intermediate and negative tested ragwed seeds in subsequent TTC tests in relation to the factors **a** age (year of harvest) and **b** origin (n = 3,600); seeds with degraded or decomposed embryos were calculated in the sum of TTC-negative tested seeds.

A regression analysis revealed that the ability to germinate and viability of the tested ragweed seeds was tightly correlated with the origin ($R^2 = 0.91$; $F = 99.17$; $p < 0.001$) and the year of harvest ($R^2 = 0.97$; $F = 65.57$; $p < 0.05$), respectively. Within years, only the seeds from Sankt Pölten (STP; 2013) showed a significant lower number of germinated seeds, but when adding the TTC-positive tested embryos to the germinated seeds (= viable) also within the year 2013, no significant differences between the origins were observed.

With the stand-alone TTC test, similar results were obtained as with the germination test (Table 6; Fig. 7). The number of positive-tested seeds was correlated with the origin ($R^2 = 0.82$, $F = 45.49$; $p < 0.001$) and the age ($R^2 = 0.84$; $F = 26.27$; $p < 0.05$), respectively. This is also true for the TTC-negative tested seeds; their number increased significantly with age ($R^2 = 0.99$, $F = 754.33$; $p < 0.001$) and was also related to the origin of the populations ($R^2 = 0.81$, $F = 43.52$; $p < 0.001$). In contrast to germination testing, it should be noted that the TTC-positive tested seeds showed generally much lower viability rates, whereas the share of intermediate coloured seeds was significantly higher (Fig. 8).

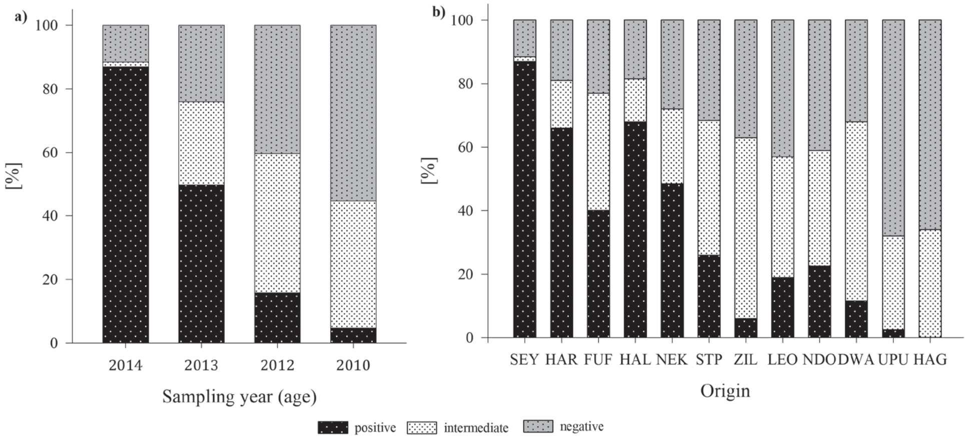

Figure 7. Share of ragweed seeds (%) tested positive, intermediate and negative in the stand-alone TTC test in relation to the factors **a** age (year of harvest) and **b** origin (n = 3,600).

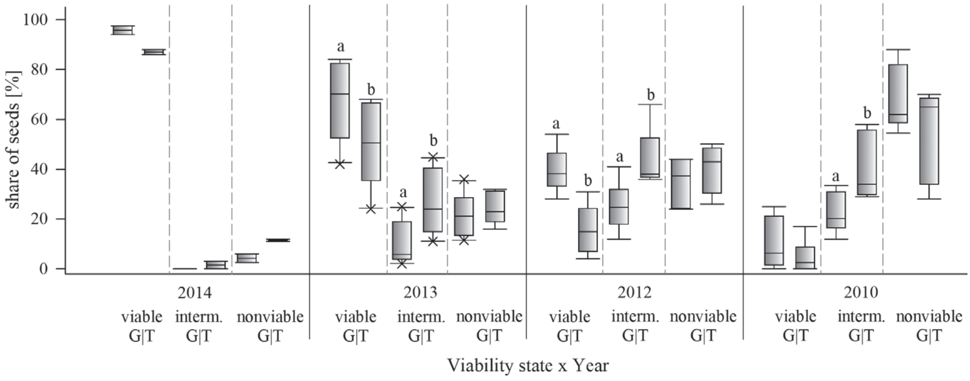

Figure 8. Results gained for the viability status of ragweed seeds estimated “viable” either by the germination test plus positive subsequent TTC test (G) or by positive stand-alone TTC test (T) dependent on the year of sampling (age); different letters indicate signficant differences between the testing methods, missing letters indicate no significant differences (2-tailed t-test; n = 7,200).

Seed weight, seed size and carbon/nitrogen-ratio (C/N-ratio)

As summarised in Table 7, we found significant differences in the seed weight of common ragweed with dependency on the factors of origin and age. The seed size (length × width) which was almost similar amongst populations did not have any impact on the results. Seeds deriving from SEY (harvested in 2014) showed an average weight of 8.8 mg ± 2.1. This was significantly higher than those of all other populations, expect that of ZIL (harvested in 2012). With an average weight of 4.7 mg ± 1.7, the lightest seeds derived from UPU (harvested in 2012). Similar seed weights were measured before the stand-alone TTC test. Results of logistic regression revealed that the factors of weight and year had significant influence on the germination rate and the viability states (stand-alone TTC test) of common ragweed seeds. However, with the germination test, we could not find any correlation between seed weight and ability to germinate. Although the youngest and heaviest seeds from SEY (2014) also showed the significantly highest germination rate, we could not detect any similar pattern amongst the other populations. For example, the average weight of common ragweed seed from ZIL (2012) was 8.0 mg ± 1.5 which did not differ from that of SEY, but the germination rate of these seeds was 73.5% lower. Similar results were obtained for the stand-alone TTC test. The highest share of positive-tested seeds (87%) was observed with seeds from SEY which had an average weight of 9.0 mg ± 1.3. However, this did not differ significantly from

Table 7. Average weight (mean ± sd) and size of ragweed seeds used in the germination test and subsequent TTC test, as well as in stand-alone tests (n = 100 seed/population), as well as the average seed weight and seed size of germinated and TTC-positive, TTC-intermediate and TTC-negative tested ragweed seeds; superscript letters indicate significant differences.

Origin	Average seed weight [mg]	Average seed size (length × width [mm]	Average weight of germinated seeds	Average seed weight [mg]	Average seed size (length × width [mm]	Average weight of TTC-positive seeds	Average weight of TTC-intermediate seeds	Average weight of TTC-negative seeds
Seyring (SEY)	8.8 ± 2.1^{a}	3.5 × 2.3	9.1 ± 1.8^{a}	8.7 ± 2.5^{a}	3.9 × 2.2	9.0 ± 1.3^{a}	–	3.2 ± 1.8^{c}
Hartberg (HAR)	6.9 ± 1.6^{b}	3.1 × 2.5	7.1 ± 1.4^{b}	7.1 ± 1.8^{ab}	3.1 × 2.4	7.6 ± 1.1^{b}	8.0 ± 0.8^{a}	4.9 ± 2.3^{abc}
Fürstenfeld (FUF)	5.7 ± 1.2^{c}	3.0 × 2.0	5.5 ± 1.3^{c}	5.6 ± 1.5^{cd}	3.1 × 2.1	5.7 ± 1.0^{b}	6.0 ± 1.5^{b}	4.3 ± 1.7^{abc}
Halbenrain (HAL)	6.9 ± 1.5^{b}	3.3 × 2.2	6.7 ± 1.2^{b}	6.7 ± 2.0^{bc}	3.5 × 2.4	7.4 ± 1.5^{b}	6.7 ± 1.5^{ab}	3.9 ± 2.1^{bc}
Neunkirchen (NEK)	6.7 ± 2.2^{b}	3.1 × 2.2	7.2 ± 1.6^{b}	6.6 ± 2.3^{bc}	3.1 × 2.2	7.0 ± 1.9^{b}	6.9 ± 1.6^{ab}	4.2 ± 2.1^{abc}
Sankt Pölten (STP)	6.6 ± 1.7^{b}	3.2 × 2.3	7.1 ± 1.6^{b}	6.7 ± 1.8^{bc}	3.1 × 2.2	6.7 ± 1.7^{b}	7.4 ± 1.9^{ab}	6.3 ± 1.7^{a}
Zillingtal (ZIL)	8.0 ± 1.5^{ab}	3.4 × 2.2	8.1 ± 1.3^{b}	7.6 ± 1.9^{bc}	3.0 × 2.1	8.2 ± 1.4^{ab}	7.3 ± 1.5^{ab}	5.9 ± 2.0^{ab}
Leobendorf (LEO)	6.8 ± 2.2^{b}	3.3 × 2.2	7.5 ± 1.8^{b}	6.4 ± 2.4^{bcd}	3.0 × 2.1	6.0 ± 2.1^{b}	6.8 ± 1.4^{ab}	3.9 ± 2.5^{bc}
Neue Donau (NDO)	6.2 ± 1.6^{b}	3.1 × 2.1	6.9 ± 1.4^{b}	6.7 ± 1.9^{bcd}	3.0 × 2.1	5.4 ± 1.1^{b}	6.9 ± 1.2^{ab}	5.1 ± 2.2^{abc}
Dt. Wagram (DWA)	6.9 ± 1.6^{b}	3.2 × 2.2	6.9 ± 1.3^{b}	5.6 ± 2.5^{cd}	3.0 × 2.1	8.0 ± 0.6^{ab}	6.6 ± 2.1^{ab}	3.6 ± 3.8^{bc}
Unterpurkla (UPU)	4.7 ± 1.7^{d}	3.0 × 2.1	–	4.5 ± 1.7^{d}	3.2 × 2.1	–	6.1 ± 1.2^{b}	3.8 ± 1.5^{bc}
Hagenbrunn (HAG)	6.0 ± 1.8^{bc}	3.2 × 2.0	–	5.4 ± 1.9^{cd}	3.3 × 2.1	–	6.5 ± 1.3^{ab}	4.9 ± 1.9^{abc}
Year								
2014	8.8 ± 2.1^{a}	3.5 × 2.2	9.1 ± 1.8^{a}			9.4 ± 1.3^{a}	–	3.2 ± 1.8^{b}
2013	6.6 ± 1.7^{b}	3.2 × 2.3	6.8 ± 1.5^{b}	6.5 ± 2.0	3.2 × 2.3	7.1 ± 1.5^{b}	6.8 ± 1.7^{a}	4.8 ± 2.1^{a}
2012	7.0 ± 1.9^{b}	3.2 × 2.1	7.4 ± 1.6^{b}	6.5 ± 2.2	3.0 × 2.1	6.2 ± 1.8^{c}	7.0 ± 1.4^{a}	5.0 ± 2.4^{a}
2010	6.3 ± 1.9^{b}	3.1 × 2.1	6.9 ± 1.3^{b}	5.4 ± 2.1	3.2 × 2.1	7.9 ± 0.6^{b}	6.4 ± 1.7^{a}	4.2 ± 1.9^{a}

the average weight of seeds deriving from ZIL (8.2 mg ± 1.4) or DWA (8.0 mg ± 0.6) which only had a share of positive-tested seeds of 6% and 11.5%, respectively. The overall mean of all positive-tested seeds amounted to 7.1 mg ± 1.2 which was similar to that of the intermediate-tested seeds (6.9 mg ± 0.6). With 4.5 mg ± 0.9, the average seeds' weight of the TTC-negative tested seeds was significantly lower.

In addition, we could not find any differences in the C/N-ratio of the seeds, ranging between 9.4 (SEY) and 11.5 (HAL). With an $R^2 = 0.09$, a regression analysis pointed out that viability of common ragweed seeds could not be correlated to the C/N-ratio (results not shown).

Discrepancies in the results with respect to the testing method

The initial Chi²-test to check for homogeneity of samples showed no significant differences; hence, it can be assumed that all samples randomly taken derive from equal populations and are, therefore, comparable. This is also true amongst testing methods comparing the seed samples used in the germination test and subsequent TTC test plus the stand-alone TTC test (Suppl. material 1: Table S2). Thus, discrepancies in the results due to unparalleled samples could be excluded. However, we found significant differences between testing results.

Figure 8 shows a comparison between the results for the viability state of the ragweed seeds between viable due to the germination test plus the subsequent TTC test (classified positive) and viable due to the positive classification in the stand-alone TTC test, compiled with respect to sampling years. Both testing methods gave comparable results for the non-viable embryos (mean over all populations of non-viable seeds' germination test plus TTC: 30.1%; TTC test alone: 34.9%). The evaluation of "viable" (germinated + subsequently TTC-positive classified seeds) and "intermediate" seeds showed significantly different results. Over all populations on average, the viability rate of the seeds accounted for 50.9% with germination test plus subsequent the TTC test, but with the stand-alone TTC test, only 33.1% of the seeds were assessed as viable (= positive) which results in a 17.8% lower viability rate.

However, a similar gap between results (13.1%) was detected when comparing the TTC-intermediate tested seeds within the germination test plus the subsequent TTC test and stand-alone TTC test: whereas the share of intermediate-stained seeds accounted for 18.9% with the germination test plus the subsequent TTC test, almost twice as many seeds (32.1%) were classified as "intermediate" with the stand-alone TTC test. For example, the greatest discrepancies were observed with seeds deriving from Fürstenfeld (FUF) which showed a 33.0% higher viability rate during germination compared to the results of the TTC test. The same was shown with the seeds from Zillingtal (ZIL) which accounted for a 32.5% higher viability rate with the germination test. Nearly exactly opposite percentage values were calculated for the intermediate seeds of these two populations, amounting to 32% for seeds from Fürstenfeld (FUF) and 28% for seeds from Zillingtal (ZIL; Table 6).

When comparing the results for the year of harvest (Fig. 8), a similar gap of "viable" and "intermediate" classified seeds between the germination test plus the subsequent TTC test and stand-alone TTC test was observed: seeds harvested in 2013 showed 20.0% more viable seeds (germinated plus fully TTC-stained seeds, respectively) with the germination test plus the subsequent TTC test than with the stand-alone TTC test. In contrast, the stand-alone TTC test showed 21.2% more intermediate seeds than the germination test plus the subsequent TTC test. In 2012, the viability rates were generally lower, but showed the same tendency: the rate of "viable" seeds was 26.7% higher with the germination test plus the subsequent TTC test, whereas the stand-alone TTC test accounted for 31.8% more intermediate seeds.

The seed staining state "intermediate"

The above figures indicate some discrepancies in the validity of "intermediate" state. It was not evident from the above figures if some of the seeds classified "intermediate" by a TTC test might be able to possibly germinate after the duration of the germination experiment. Table 8 summarises the results of the statistical analysis to calculate the probability on an intermediate stained seed to be viable (= able to germinate) or not. This analysis was performed on the basis of the results of the germination test plus the subsequent TTC test and stand-alone TTC test of the twelve Austrian seed lots of the extended trial. It should indicate that the probability of intermediate seeds to be viable is evident in eleven out of twelve populations independent of their age and

Table 8. Probability of ragweed seeds of intermediate status to be viable or non-viable verified by a Chi^2 -test: Col. 2: comparison number of viable (germinated + TTC-positive) seeds with the number of non-viable seeds without consideration of intermediate seeds; Col. 3: comparison of numbers of viable seeds and non-viable seeds, intermediate seeds were counted as non-viable; Col. 4: comparison of number of viable and non-viable seeds, intermediate seeds were counted as viable (n = 7,200); significance levels: * p < 0.05, ** p < 0.01, *** p < 0.001 indicate probability if seed is viable or not.

Population	p-valuesfor the similarity of results of the germination test plus subs. TTC test and stand-alone TTC test (without intermediate seeds)	p-value for the probability of stand-alone TTC-tested intermediate seeds to be non-viable	p-values for the probability of TTC-intermediate seeds to be viable
Seyring (SEY)	0.05296	0,02753 *	0,05700
Hartberg (HAR)	0.26399	0.01625 *	0.48152
Fürstenfeld (FUF)	0.28149	< 0.001 ***	0.74054
Halbenrain (HAL)	0.14885	0.01366 *	0.26280
Neunkirchen (NEK)	0.29086	0.00901 **	0.90559
Sankt Pölten (STP)	0.13935	0.00499 **	0.87951
Zillingtal (ZIL)	< 0.001 ***	< 0.001 ***	0.85522
Leobendorf (LEO)	0.00119 **	< 0.001 ***	0.15576
Neue Donau (NDO)	0.03178 *	0.00935 **	0.30658
Dt. Wagram (DWA)	0.83300	0.03839 *	< 0.001 ***
Unterpurkla (UPU)	0.64976	0.54974	0.27286
Hagenbrunn (HAG)	0.08548	0.06912	0.44658
OVERALL	**0.26142**	**0.02538 ***	**0.92871**

origin. Comparing the viability statistics over all populations, the probability of a seed classified as intermediate to be viable is 92.8% in this series of experiments (Col. 4). If intermediate seeds were not considered (Col. 2), particularly for the populations from Zillingtal (ZIL), Leobendorf (LEO) and Neue Donau (NDO), a great divergence between the results gained in the germination test plus the subsequent TTC test and in the stand-alone TTC test was observed, due to the highest number of intermediate seeds in just these populations. However, if these intermediate seeds were counted as viable, the results showed that the probability of these seeds to be viable is significantly higher than being non-viable. However, with increasing age, this classification was biased. All intermediate seeds that derived from populations harvested in the year 2010 (DWA, UPU, HAG) could not be classified since the probability of an intermediate seed to be viable or not was indifferent within this populations.

Discussion

Germination and Viability

The results of both trials showed that origin and age had a significant impact on the viability of ragweed seeds, irrespective if tested with the germination test or TTC test. In the joint trial, the youngest seeds H1-2014 showed a germination rate of 74.7%, which was twice as high as those of H2-2011. In addition, the share of TTC-positive tested seeds was 78.8%, almost double that of the seeds harvested in 2011 (44.9%).

Similar results were obtained with the extended trial - age and origin of the ragweed seeds influenced germination rate significantly. Results showed clearly that, with increasing age, the germination capacity declined sharply from 96.0% to 8.8% within 5 years, the share of viable TTC-tested seeds decreasing from 87.0% to 4.7% (2014 vs. 2010).

A distinct decrease in the viability of common ragweed seeds has already been proved by Karrer (2016) and Kazinczi and Kerepesi (2016), based on seeds stored at 4 °C or at room temperature, respectively. Our results are in accordance with Harrison et al. (2007) who investigated the demise of the seeds of a congener of common ragweed, *Ambrosia trifida* (giant ragweed) under cold dry storage. In this study, the germination capacity also declined from approximately 70% to 0–19% within a four years range.

Furthermore, significant differences within years and between the different origins, respectively, were observed with both testing methods. Thus, only seeds collected in the same year should be used for analysing effects of other factors.

Even though numerous studies already showed that traits like seed weight and seed size could play a vital role in germination behaviour of various plant species (Souza and Fagundes 2014; Kumar et al. 2017; Yi et al. 2019), we did not find clear evidence for that. For example, in the joint test, the lowest germination rate and the lowest share of TTC-positive tested seeds was observed with the seeds from A1-2012 which had the highest 100 kernel weight. In contrast, in the extended trial, the highest germination rate and the highest share of TTC-positive tested seeds were observed with the youngest and heaviest seeds deriving from SEY. Thus, there was no clear correlation between seed weight and viability detectable. This is in accordance with Guillemin and Chauvel (2011) and Ortmans et al. (2016) who also concluded that seed weight had no influence on the seed viability of common ragweed. Similar, contradictory results were gained when observing other Asteraceae species like *Packera tomentosa* (Leverett & Jolls, 2014), implying that seed heteromorphism is not related to particular species, but very prominent within the Asteraceae-family and is influenced, not only by biotic and abiotic parameters like climatic and competitive conditions, but also by genetic parentage.

The same is true for the C/N-ratio which did not have any impact on the results. For example, the most viable seeds which were harvested in 2014 in SEY showed the highest N-percentage, but this did not differ significantly from the "weakest" population sampled in HAG in 2010. Viability studies on crops and different weedy species like *Amaranthus retroflexus* already showed that germination can be accelerated or decelerated by the N-content of seeds (Holdsworth et al. 2008; Karimmojeni et al. 2014). However, we could not find an influence of the initial N-content of common ragweed seeds on its ability to germinate.

Mean germination time

As with the germination rate, the MGT was strongly affected in both, the joint trial and the extended trial by the factor year, indicating that younger seeds germinate significantly faster than older seeds. However, even though the participating labs within the joint trial were using a standardised protocol, significantly different MGTs between labs were observed. To ensure the traceability of the results, the participants were provided with a blank form in the run-up to the joint-trial for submitting the results, but which also contained questions on storage and incubation conditions, as well as questions on the monitoring practice. Unfortunately, not all participants used this form for submitting the data. It is, therefore, quite difficult to understand these severe discrepancies in the MGT, even though - on request - all participants confirmed the correct application of the germination protocol.

With the extended trial, significant differences within years and between origins, respectively, were also observed, especially between the oldest seeds collected in the year 2010 which also showed the lowest germination rates. The amplitude of temperature-, moisture- and light conditions for germination following stratification is usually broad for common ragweed seeds in secondary dormancy (Baskin and Baskin 1998). Thus, the germination rate and the temporal distribution of germination can be strongly influenced by the temperature and light regime, especially when seeds were stored under controlled conditions (Baskin and Baskin 1980; Dinelli et al. 2013; Farooq et al. 2019). This underlines the importance of subsequent viability tests after the germination test which commonly takes 28 to 30 days and can often not be extended due to resource and time limitations.

Overestimation of viability in crush-test

The crush test is a widely accepted means of testing seed viability and various studies have shown that results of the crush test are comparable with other means like TTC testing (Sawma and Mohler 2002; Borza et al. 2007). Controversially, in the present study, the crush test led to a significant overestimation of viable seeds. On average, 55.6% more seeds were tested viable with the crush test when compared with both other testing methods. An explanation for this severe discrepancy could be the fact that seeds and embryos, respectively, of common ragweed are not only dead (no germination or discolouration in TTC) or empty (no embryo in the seed), but can also show indications of decomposition or degradation. When crushing these non-intact seeds, they also release moisture, which could be interpreted as liquid staining, hence leading to a misclassification as viable. Crush-test results indicate a false estimate of ragweed seed viability and should not be applied for scientific analyses.

Germination vs. TTC Test

In general, we can state that all germinated seeds can be classified viable, but some other viable seeds do not germinate due to seed dormancy. Viable seeds comprise of the germinated seeds plus the TTC-positive seeds anyway. This number of viable should be ± identical to the number of TTC-positive seeds. Even though testing viability by using TTC is common practice in broad fields of plant and microbiological research (AOSA 2000), significant differences between the results of the simple germination test and the TTC test were observed, particularly between the number of germinated seeds and the number of seeds tested positive and intermediate, respectively, with the TTC test. One possible reason for these discrepancies could be the categorisation of the state “intermediate” since this state covers a wide range of different colouration intensities (Fig. 9). The colouration might be addressed to differing viability stages (Karrer et al. 2016b). Thus, it is still unclear if these seeds are still viable or not and if there is a so-called threshold of colouration, below which seeds could be categorised as dead and beyond this, seeds would still be able to germinate. Since germination tests with these TTC-treated seeds are no longer feasible, statistical analysis was performed on the basis of the results of germination tests and TTC tests of the same seed cohorts, showing that the possibility of a TTC-intermediate tested seed to be viable was 92.87%, calculated over all populations and seed ages. Despite that high probability value, it was evident that, with increasing seed age, results were biased since for all intermediate tested seeds from the year 2010, a proper statistical classification was not possible, as the probability of the intermediate seeds to be viable or not was indifferent. Summarising, it could be assumed that the probability of a seed tested TTC-intermediate to be viable decreases with increasing seed age which is in accordance with numerous studies on seeds of different species (Bewley and Black 1982; Baskin and Baskin 1998; Walters et al. 2005; Harrison et al. 2007).

Seeds classified unanimously as viable, intermediate or negative

Seeds classified as viable, intermediate or dead

Seeds classified as viable or intermediate

Seeds classified as intermediate or dead

Figure 9. Examples of microscope pictures of seeds after TTC colouration classified by the joint-trial participants in the post-experiment evaluation with different accurrancy.

Differences between labs

With the joint trial, severe discrepancies in the evaluation of TTC-stained seeds were detectable, especially amongst labs, but not within labs when two independent testers were employed. However, even though the participants were provided with a standardised protocol giving information on testing procedures, evaluation practice, as well as storage and incubation conditions, not all participating labs followed these specifications. For example, in the Romanian lab, seeds were only incubated in TTC solution for 12 hours instead of the proposed 24 hours in the protocol. This could explain why both testers of the Romanian lab counted the significantly lowest number of fully-coloured (TTC-positive) seeds, whilst their share of TTC-intermediate coloured seeds was highest amongst all labs.

Another reason for these differences in the results could be a certain degree of uncertainty, particularly with the TTC-state “intermediate” (Figs. 1 and 9). For a proper classification of the seeds, the embryos have to be pulled out of the involucrum and the achene and have to be checked under a microscope since colouration and non-colouration, respectively, are often discreet and, therefore, not visible to the naked eye. If this is not done, the risk of classifying a seed as “positive” or “negative” instead of intermediate is quite high, as was the case in the Serbian lab. Over all populations, only 0.5% of the seeds were classified as intermediate due to improper evaluation practice.

Generally, it should be noticed that classification of TTC-stained seeds is, to some extent, due to subjectivity since the three different states are not always clearly divisible (Karrer et al. 2016b). To check the impact of subjectivity, subsequently to the joint trial, 50 microscopic pictures of TTC-stained seeds were sent to the participants of the joint trial who were ask to evaluate whether the seeds are positive, intermediate or negative. Results (Fig. 9) showed clearly that subjectivity has an impact to a very large extent. Only with 17 out of 50 seeds, a unanimous result was given. With 12 seeds, all states (positive, intermediate and negative) were represented and the remaining 31 seeds were either classified as positive or intermediate and intermediate or negative, respectively (Fig. 9).

Conclusions

Viability of common ragweed seeds is strongly influenced by age and origin. However, various environmental factors (light conditions, temperature, nutrient availability, soil type etc.), as well as storage conditions, have to be considered when testing for viability of seeds. Particularly with joint trials, this study clearly reveals the problems involved in such ring-experiments. On the one hand, some results were not traceable even though participants were provided with standardised protocols and forms. On the other hand, subjectivity in evaluating results led to significant discrepancies amongst labs. As a consequence, the aim is to develop improved protocols and evaluation standards, especially for TTC testing to ensure that future joint tests show better comparability and traceability of results. This study, therefore, contributes to the improvement of testing standards for estimating the infestation rate of any containment with common ragweed. Germination tests plus the subsequent TTC tests of the remaining seeds gave almost the same number of viable seeds anyway and is, therefore, the most reliable testing method. Thus, we can recommend both test strategies because of ± equal validity. If time is short, the stand-alone TTC test achieves sufficient validity if subjectivity in colours' interpretation is reduced. The crush test only gives not really valid estimates of viable common ragweed seeds. Furthermore, we would like to underline that the testing labs should strictly follow the actualised guidelines (i.e. Karrer et al. 2016c).

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Supplementary material 1

Supporting tables and figures

Authors: Rea Maria Hall, Bernhard Urban, Hana Skálová, Lenka Moravcová, Ulrike Sölter, Uwe Starfinger, Gabriela Kazinczi, Johan van Valkenburg, Annamaria Fenesi, Bojan Konstantinovic, Ahmet Uludag,Suzanne Lommen, Gerhard Karrer

Data type: tables and figures

Explanation note: **Table S1.** Results of the ANOVA showing significant differences among labs (incl. tester-code) with the results of the stand-alone TTC-test on ragweed seed viability of Austrian seed lots (percentages of TTC-positive, TTC-intermediate and TTC-negative per population); different letters indicate significant differences between the seed lots within the respecive staining class. **Figure S1.** Germination rate [%] of ragweed seeds in relation to the factors origin and age (n = 1,536; different letters indicate significant differences between origins; codes see in Tab 4). **Table S2.** Results of Chi^3-Test: Probability that randomized samples for germination test plus subsequent TTC-test (Col. 1) and stand-alone TTC-test (Col.2) as well as all samples taken (Col. 3) originate from same overall populations (n = 7,200); as indicator values the number of germinated and/or TTC-positive tested seeds were used.

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