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Contact rates with nesting birds before and after invasive snake removal: estimating the effects of trap-based control

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Abstract

Invasive predators are responsible for almost 60% of all vertebrate extinctions worldwide with the most vulnerable faunas occurring on islands. The brown treesnake (*Boiga irregularis*) is a notorious invasive predator that caused the extirpation or extinction of most native forest birds on Guam. The success of avian reintroduction efforts on Guam will depend on whether snake-control techniques sufficiently reduce contact rates between brown treesnakes and reintroduced birds. Mouse-lure traps can successfully reduce brown treesnake populations at local scales. Over a 22-week period both with and without active snake removal, we evaluated snake-trap contact rates for mouse- and bird-lure traps. Bird-lure traps served as a proxy for reintroduced nesting birds. Overall, mouse-lure traps caught more snakes per trap night than did bird-lure traps. However, cameras revealed that bird-lure traps had a snake contact rate almost 15 times greater than the number of successfully captured snakes. Snakes that entered bird-lure traps tended to be larger and in better body condition and were mostly captured in bird-lure traps, despite numerous adjacent mouse-lure traps. Traps placed along grid edges caught more snakes than interior traps, suggesting continuous immigration into the trapping grid within which bird-lure traps were located. Contact between snakes and bird-lure traps was equivalent before and after snake removal, suggesting mouse-lure traps did not adequately reduce the density of snakes that posed a risk to birds, at least at the timescale of this project. This study provides evidence that some snakes exhibit prey selectivity for live birds over live mouse lures. Reliance on a single control tool and lure may be inadequate for support of avian reintroductions and could lead to unintended harvest-driven trait changes of this invasive predator.

Keywords
Avian recovery, biological invasions, brown treesnakes, control, Guam, restoration

Introduction

Invasive predators are a major driver of vertebrate extinctions globally (Szabo et al. 2012; Doherty et al. 2016). Although mammalian predators are the primary cause (Doherty et al. 2015), invasive snakes have also been linked to extinctions and extirpations of native vertebrates (Savidge 1987; Dorcas et al. 2012). Reducing or eradicating invasive predators can lead to recolonization of nesting sites (Borrelle et al. 2016), increased prey populations (Campbell et al. 2012), and recovery of native species (Jones et al. 2016). In a systematic worldwide review, predation by non-native predators was identified as one of the leading drivers for failed reintroductions (Destro et al. 2018), where reintroduction is defined as a type of conservation translocation that entails deliberate releases of individuals within their native range where they have otherwise been extirpated (IUCN/SSC 2013). For native species that have been locally extirpated or driven to extinction in the wild in part by predation by invasive predators, removal or reduction of non-native predators is essential (Choquenot and Parkes 2001; VanderWerf et al. 2014). Therefore, when invasive predators are present, management and suppression of their populations is often a component of native species recovery plans (U.S. Fish and Wildlife Service 2006; IUCN/SSC 2013) and may be critical when recovery of native species requires reintroduction efforts (Côté and Sutherland 1997; Smith et al. 2010).

In regions where biodiversity is affected by invasive predators, core components of invasive predator control include exclusion, shooting, trapping, and toxicant baiting (O'Donnell et al. 2017). Because species recovery is often linked to predator control, assessing the impact of such programs on the anticipated interactions between non-native predators and the species targeted for recovery is crucial for population restoration (Choquenot et al. 2001). Prioritizing these evaluations during pre-release planning or post-release monitoring may increase the success of reintroduction programs (Destro et al. 2018), encourage adaptive management, and allow refinement of control tools and lures (Klug et al. 2015).

Guam, the southernmost island in the Mariana Archipelago, experienced major biodiversity loss after the introduction of the non-native brown treesnake, *Boiga irregularis*, after World War II (Savidge 1987; Wiles et al. 2003). This accidental introduction resulted in high snake densities across the island and caused the extirpation of many native terrestrial vertebrates, with 10 of 12 forest bird species eliminated (Savidge 1987; Wiles et al. 2003; Rodda and Savidge 2007). Declines and reductions of bird populations on Guam are suspected to be causing major ecological changes to forest structure and composition (Rogers et al. 2017). Therefore, reintroducing, reinforcing, and otherwise recovering native bird populations on Guam are considered a critical goal for broader restoration targets. Suppression and removal of invasive brown treesnakes are a critical management need for recovery of Guam's native birds.

Localized brown treesnake control on Guam to reduce snakes at seaports, airports, and caves used by Mariana swiftlets (*Aerodramus bartschi*) has historically relied on removal primarily using mouse-lure traps (Rodda et al. 1999; Clark et al. 2018) but experimental aerial application of toxicant baits (dead neonatal mouse with acetaminophen tablet) to suppress snake populations is emerging as a potential landscape-scale control tool (Dorr et al. 2016; Siers et al. 2019). Mouse-lure traps can remove every individual of suitable size (≥950 mm snout-vent length) at a small spatial scale, given intensive effort (Tyrrell et al. 2009). The effect of extensive snake trapping on predation rates on birds (and therefore the likelihood of successful bird restoration efforts) has not been quantified. We therefore evaluated the potential benefits of trap-based snake removal for bird reintroductions by simulating the avian nesting period because that aspect of the life-cycle is vulnerable for birds (Martin 1993; Yackel Adams et al. 2006). Specifically, we 1) measured the fraction of trap contacts that resulted in a snake capture, 2) compared brown treesnake contact rates between mouse- or bird-lure traps, and 3) compared contact rates between bird-lure traps (used as a proxy for reintroduced nesting birds) and brown treesnakes before and after active snake control (trapping with mouse lures). The results are interpreted in the context of brown treesnake management to understand the actual complexities inherent to reintroductions or reinforcements of native bird populations on Guam.

Methods

Study site

The study occurred in the Ritidian Unit of the Guam National Wildlife Refuge (GNWR; 13°39'N, 144°51'E), at the northernmost tip of Guam. The 155 ha terrestrial portion of the refuge consists of coastal strand forest interspersed with degraded areas that have been colonized by non-native shrubs and trees (U.S. Fish and Wildlife Service 2009). The site is bordered to the south by limestone cliffs and to the north by the Pacific Ocean. In 2012, a multi-species barrier fence was constructed around 51 ha of the Ritidian Unit; this fence included a one-way barrier designed to exclude snakes while allowing snakes on the refuge to leave the enclosed area (Rodda et al. 2007). Snake control efforts were implemented in 2013 through use of mouse-lure snake traps (15,447 trap nights that removed 392 snakes) to suppress brown treesnakes within the barrier at the GNWR. Based on that control effort, GNWR was considered a snake-suppressed area (Nafus et al. 2018) and was used to measure contact rates between birds and brown treesnakes. Snake density within the barrier was unknown at the time of the 2013 removal effort, but 23 snakes per hectare has been documented in a nearby enclosed forested habitat (Christy et al. 2010). If we assume that density estimate along with the assumption that every hectare within the refuge barrier is suitable snake habitat, then we have a snake population of 1,173 (23 snakes/ha × 51 ha). This rough estimate indicates that one-third of the snake population may have been removed during the 2013 removal effort. Although the snake barrier had by 2014 partially degraded (i.e., small patches of rusted fencing due to salt spray) and probably allowed some immigration, the barrier was mostly intact and abutted a road. Brown treesnakes avoid crossing roads (Siers et al. 2016) and thus the road may have hindered snake crossing into GNWR, improving the efficacy of the barrier. Limited and sporadic rat and feral pig control measures were implemented within the barrier.

Trapping array and capture rates

In May 2014, we established a 6 × 18 trapping grid (510 m × 150 m; Fig. 1a) of 108 live mouse-lure traps (Fig. 1a [yellow dots] and Fig.1b) with 16 live bird-lure traps (Fig. 1a [red and blue dots] and Fig. 1c). We used Japanese quail (*Coturnix japonica*) in place of a native bird species. Japanese quail are neither present in the wild on Guam nor a federally/territorially listed species, but likely functionally equivalent to Guam rail (*Gallirallus owstoni*) in terms of prey odor and habitat strata use. Bird-lure traps thus served as a proxy for a reintroduced population of nesting birds and were set 2 weeks prior to the mouse-lure traps (Phase I) to obtain baseline predation rates in the absence of active snake control (Phases I and II). After 2 weeks, we added mouse-lure traps, but continued to trap without removing snakes for 60 days (Phase II). Beginning on 07 July 2014 (day 61) we removed all snakes captured in mouse-lure traps to monitor contact rates during active suppression efforts (Phase III). Snakes trapped in bird-lure traps were, however, never removed to simulate realistic snake contacts with nesting birds.

Mouse-lure and bird-lure traps are modified commercial minnow traps composed of 6 mm galvanized steel mesh (Rodda et al. 1999; Fig. 1b, c). Each mouse-lure trap contained a lure chamber and PVC pipe refuge for trapped snakes. Lure chambers were constructed of galvanized steel mesh and held a single mouse (20–40 g) that was provided a grain mixture embedded in paraffin and a piece of raw potato for water. Bird-lure traps were modified versions of the commercial minnow trap, with a central extension to provide room for the birds (Fig. 1c). Bird chambers (35 × 13 × 17 cm, LWH) inside the traps were constructed of galvanized steel mesh (6 mm). Birds (150–180 g) were provided a pellet seed mixture, millet sprig, and water. Both trap types allowed multiple snake captures. Bird-lure traps were checked daily and mouse-lure traps checked every other day. Mouse-lure traps were stationary during the study (to mimic operational control efforts) whereas the two lines of bird-lure traps moved weekly to the next available grid space, to sample a larger percentage of the grid. For instance, bird-lure traps in week 1 (configuration shown in Fig. 1a) deployed between the transect lines of D and E, and B and C would move north (toward the ocean) one grid space in week 2 to occupy grid locations between C and D and A and B, respectively.

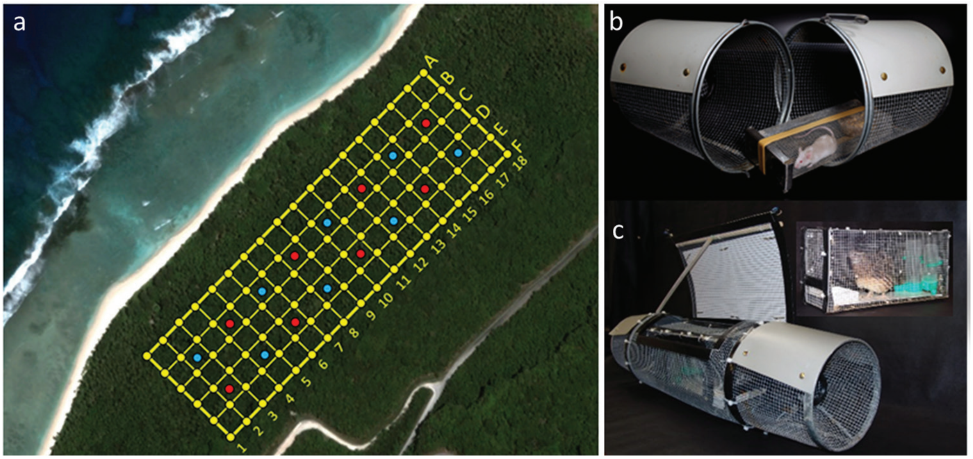

Figure 1. The 7.65 ha grid consisted of 124 traps (**a**). Yellow dots represent mouse-lure traps (*n* = 108 traps) whereas red and blue dots represent bird-lure Japanese quail (*Coturnix japonica*) traps (n = 16) with and without cameras, respectively. Mouse-lure traps (**b**) were spaced every 30 meters and were stationary. Bird-lure traps (**c**) were spaced at 60 meters and were moved weekly to a new location. For instance, bird-lure traps in week 1 (configuration as shown in **a**) would move in week 2 from the alpha transect lines of BC and DE to AB and CD, respectively. New locations would remain in the same numeric transect lines of 2–3, 4–5, 6–7, 8–9, 10–11, 12–13, 14–15, and 16–17 (until all interior spaces had been sampled) before shifting in week 6 to numeric lines of 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, 15–16, and 17–18. Each bird-lure trap location was sampled two times for a total of two weeks. The snake barrier fence runs along curved road line (bottom right of panel a). Photo credit for 1b: Shane R. Siers.

To quantify the proportion of snake-bird contacts that failed to result in trap captures, eight of the bird-lure traps were fitted with trail cameras at a 1.8 m focal distance (Reconyx PC 900 HyperFire Professional covert camera; Fig. 1a [red dots]). We programmed cameras with both time-lapse (30-sec intervals between the hours 1800 and 0600 [brown treesnakes are nocturnal; see Suppl. material 1 for example camera images]) and motion sensor modes (any time of day). Cameras were placed 1.8 m from the focal trap and batteries and SD memory cards were changed every 3 days. Digital images were downloaded and transcribed to record all snakes visible in the camera field of view (FOV) as well as other potential predators. Brief absence from FOV, return to FOV in close spatial proximity to FOV departure location, similar physical attributes [broken tail, size] were counted as a single snake. Trap contact consisted of the snake making physical contact with the trap.

Snake morphometrics

Unless destined for removal (during Phase III), we marked trapped snakes on the first occasion we encountered them, before re-releasing them at the site of capture. Marking consisted of a passive integrated transponder (PIT) tag injected intraperitoneally, and a unique series of ventral scale clips. Measurements of mass and snout-vent length (SVL) were recorded for each snake capture. Individual body condition was calculated as the ratio of mass to its expected mass given its length. Expected mass for a given SVL was estimated by linear regression on logarithmic scales, based on >10,000 records of brown treesnakes. Snakes that we removed (Phase III: active control, mouse-lure traps) were euthanized using procedures approved by the American Veterinary Medical Association (2013) and USGS Fort Collins Science Center, Institutional Animal Care and Use Committee (FORT IACUC 2013-13).

Statistical analyses

We used Poisson regression to test the effect of lure type (bird or mouse) on catch per unit effort (CPUE) during Phase II of the project when both bird- and mouse-lure traps were present on the landscape, but snake removal was not occurring. CPUE was measured as the number of snakes captured per 100 trap nights, where a trap night is defined as 1 trap active for 1 night. We used multivariate multiple regression to measure the effect of trap lure type and time since project initiation on SVL and body condition. We included both SVL and body condition as dependent variables in the model. Although we were primarily testing for the effect of bird-lure versus mouse-lure traps as a predictor of SVL and body condition, we included time (days) since project initiation as a covariate due to changes in snake population structure that can result from active removal or seasonal effects. We used Pearson's chi-square to test for a change in contact rates between snakes and bird-lure traps or cameras after the onset of snake removal. For the chi-square we compared camera and trap CPUE (snakes per 100 days of trapping) prior to active removal to CPUE after trap-based removal began. Finally, we used mixed-effect, zero-inflated Poisson regression (GLMMADMB package in R) to test for differences in snake capture rates between mouse-lure traps near a bird-lure trap and those not near one, as well as for differences between grid edge versus interior mouse-lure traps. We included alpha trap transect lines (A–F; Fig. 1a) as a random effect to account for repeated measures and spatial variation in trap captures. In Figure 1a, all grid interior mouse-lure traps were considered adjacent to a bird-lure trap and all grid edge traps as non-adjacent. Interior and edge classification would change weekly as the bird-trap deployment was altered by weekly trap placement (defined above). All analyses were executed in program R (R Core Team 2017) and descriptive statistics reported as mean ± SE.

Results

Over the course of the study (08 May to 05 Oct. 2014), we recorded 159 unique snakes from 227 captures during 16,947 trap nights (0.013 snakes/trap night). Females (*n* = 82) averaged 1035 mm SVL (range 688–1,265; body condition = 1.15, range 0.82–1.54). Males (*n* = 77) averaged 1081 mm SVL (range 773–1,400; body condition= 1.07, range 0.71–1.39). Of the 227 captures, 198 snakes were captured in mouse-lure traps (134 individuals; 0.014 snakes/trap night) and 29 were captured in bird-lure traps (25 individuals; 0.012 snakes/trap night).

Camera and trap CPUE

In order of prevalence, surveillance cameras deployed on eight bird-lure traps captured 2,314 FOV incidents from feral pigs (1,727), snakes (307), rats (228), monitor lizards (44), and cats (8). Of the 307 FOV records for snakes, 217 snake encounters were considered independent snakes for that evening. Fifty-six percent (122 of 217) of the images revealed a trap contact by the snake, suggesting interest in the bird lure. Overall snake CPUE at camera traps was 0.18 (Fig. 2), yielding a contact rate of 18 snakes/100 camera-trap nights. However, only 13% of trap contacts resulted in a trap capture (Fig. 3). Cumulatively, bird-lure traps captured 29 snakes across 2,321 total trap nights (1.2/100 bird-lure trap nights). Thus, trap captures underestimated the trap-contact rates with birds 15-fold relative to trap-contact rates estimated by camera traps. Snakes that successfully entered the bird-lure traps spent on average 55 min to enter (8 min up to 2 hours and 23 min). Snakes that failed to gain entry to the trap gave up and departed the FOV on average after 17 min (30 s to 50 min).

Snake contact rates with bird- and mouse-lure traps

During Phase II, when both bird- and mouse-lure traps were deployed but no active snake removal occurred, we recorded 732 bird-lure trap nights and 4,942 mouse-lure trap nights. Bird-lure traps captured six snakes (0.8 snakes/100 bird-lure trap nights) and mouse-lure traps captured 69 snakes (1.4 snakes/100 mouse-lure trap nights). A small portion of snakes (14%) were repeatedly captured in mouse-lure traps (≥ 2 times) and almost all unique captures during Phase II were snakes only captured in mouse-lure traps (95%). Mouse-lure traps had a CPUE that was 1.7 times greater than bird-lure traps based on Poisson regression (z = 4.1, $P < 0.001$, 95% Confidence Interval [CI] = 0.29, 0.82, Fig. 2).

The 25 unique snakes captured in bird-lure traps averaged 26 mm longer and 19 g heavier than snakes captured in mouse-lure traps (Table 1). Mean body condition for snakes captured in bird-lure traps was 1.16 ± 0.03 and 1.12 ± 0.01 for mouse-lure traps (Table 1). Multivariate multiple regression indicated a weak negative relationship between mouse-lure traps and SVL and body condition of snakes captured (t = –2.0, $P = 0.04$, 95% CI = –0.186, –0.002) and a negative effect of time since project initiation (t = –2.2, $P = 0.02$, 95% CI = –0.0016, –0.0001). Although

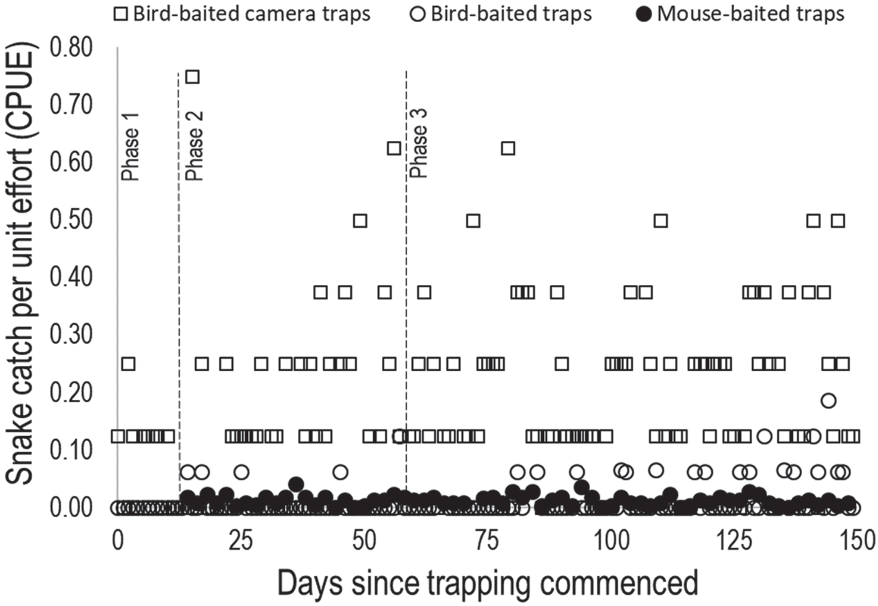

Figure 2. Brown treesnake catch per unit effort (CPUE) per trap night for bird-lure camera traps and bird- and mouse-lure live traps from 08 May through 05 October 2014 on the Guam National Wildlife Refuge, Guam. Open squares represent capturing a photographic image of the snake. Open and closed circles represent actual successful snake captures from traps. Phase I = only bird-lure traps deployed, Phase II = both bird- and mouse-lure traps deployed, and Phase III = both bird- and mouse-lure traps deployed with snake removal from mouse-lure traps only. Cameras were deployed on bird-lure traps during all three phases.

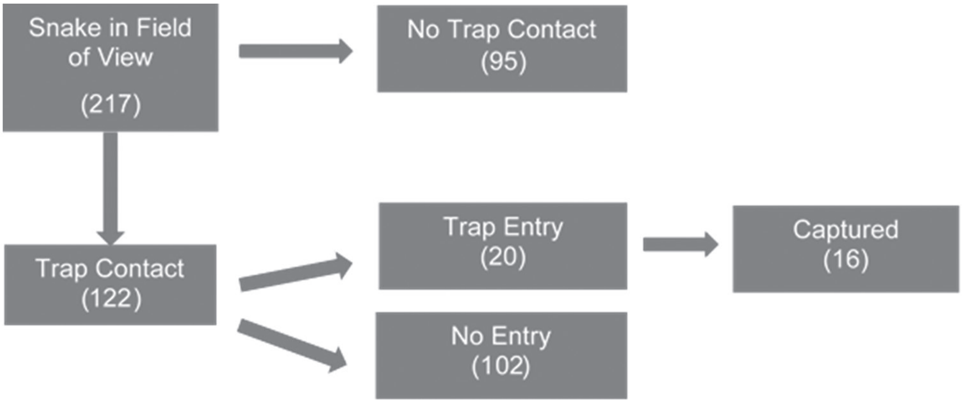

Figure 3. Schematic of brown treesnake activity outcomes at bird-lure camera traps (*n* = 8). A portion of snake observations were probably repeated instances of one snake's efforts to capture the prey (e.g., brief absence and return to field of view in close spatial proximity to departure location and similar physical attributes [broken tail, size]) and were therefore counted as a single snake event. Trap contact consisted of the snake making physical contact with the trap. Trap entry consisted of snakes using either entrance to enter the trap. Values listed parenthetically represent the number of snakes for a specified outcome, with snakes captured in traps being the desired outcome for management.

Table 1. Morphometrics of individual brown treesnakes (*Boiga irregularis*) trapped with live mouse- and/or live bird-lures based on first encounter presented as mean ± SE (range), Guam National Wildlife Refuge 2014.

Lure	BC[1]	BC range	SVL (mm)	SVL range (mm)	Mass (g)	SVL>1150 (mm)
Bird-lure *n* = 25	1.2	0.89–1.46	1091	885–1304	160 (60–352)	35%
Mouse-lure *n* = 140	1.1	0.71–1.66	1065	688–1400	141(29–435)	25%

[1] Body condition (a value of < 1 represent relatively underweight individuals, average condition snakes = 1, and higher than average are > 1).

some individuals were repeatedly captured in bird-lure traps, 20 of the 25 unique bird-lure captures (80%) were only captured in bird-lure traps. Three individuals (12%) were captured more than once in bird-lure traps, but never in mouse-lure traps. Five individuals were captured in both trap types and were removed during Phase III, suggesting 20% of snakes that entered a bird-lure trap were effectively removed by mouse-lure trapping.

Contact rates with traps pre- and post-removal

During Phase III, we removed 128 snakes from the trap grid using mouse-lure traps. Despite removal, overall daily CPUE of snakes in mouse-lure traps remained constant but low (1.4 snakes/100 mouse-lure trap-nights). Camera trap CPUE at bird-lure traps prior to snake removal (Phases I and II) was 14 snakes/100 camera-trap nights and 19 snakes/100 camera-trap nights after snake removal began. Trap CPUE for bird-lure traps was 0.6 snakes/100 bird-lure trap nights prior to snake removal and 1.7 snakes/100 bird-lure trap nights after snake removal began (translating to 1.3 snakes/100 bird-lure trap nights overall). There was no significant effect of snake removal and snake contact with birds (χ [1] = 0.20, $P = 0.65$) in Phase III as compared to Phases I and II. Overall, the number of consecutive days without a capture (n = 29 snakes) in a bird-lure trap decreased over time despite snake removal and weekly movement of bird-lure traps from 10.4 days during the first 5-week interval to 1.6 days during the last 5-week interval (Fig. 4a).

There were also spatial effects on snake captures independent of snake removal. Almost half of the 29 bird-lure captures occurred between trap lines E and F (Fig. 1a), and mouse-lure captures on line F also had the highest CPUE (Fig. 4b). Although mouse-lure traps did not appear to suppress bird-trap contact rates, they did suppress contact rates with mouse-lure traps. Mixed-effect Poisson regression indicated approximately 50% fewer snakes were captured in traps deployed in the grid interior relative to edge traps ($\beta = -0.67$, SE = 0.21, $P = 0.002$). Across 20 weeks of mouse-lure trapping, grid edge traps captured 80 snakes (44 edge traps), compared to 60 snakes captured in interior traps (64 interior traps). Mouse-lures near bird-lures, however, had the same CPUE as those that did not have a bird-lure present ($\beta = -0.10$, SE = 0.21, $P = 0.65$). Therefore, captures of snakes attracted to mouse lures were depressed in the grid interior but there was no evidence for a similar effect for bird lures.

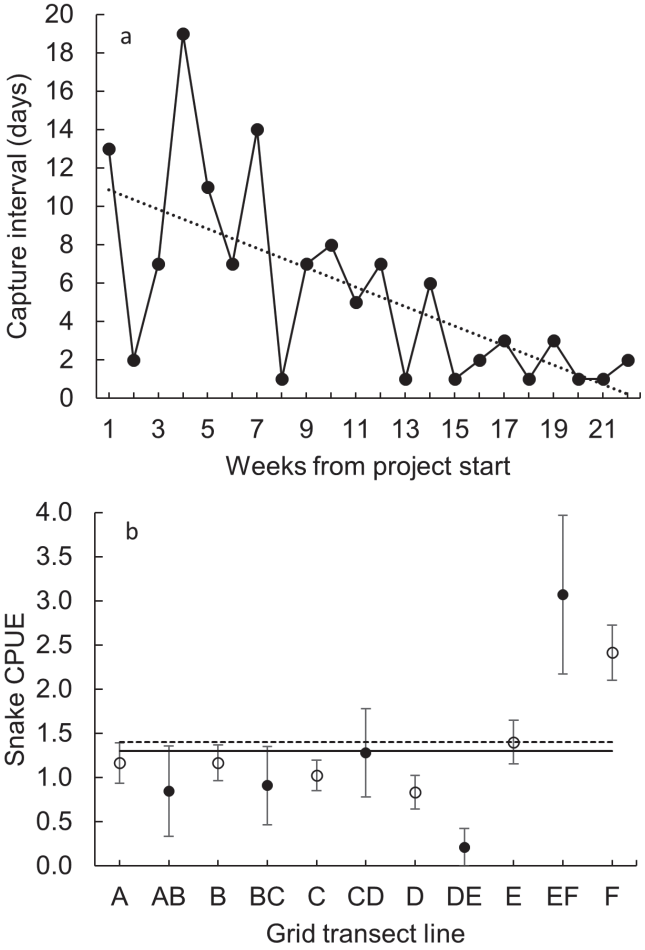

Figure 4. We observed temporal (**a**) and spatial (**b**) effects on brown treesnake captures at bird- and mouse-lure traps (note: graphs do not include camera data). Capture intervals (days between capturing any snake in a bird-lure trap) decreased as length of time from study start date increased (**a**). Catch per unit effort (CPUE, snakes/100 trap nights) was greater for bird-lure (closed circles) and mouse-lure (open circles) transects that were closer to the cliff-line (E–F; see **b**). In panel b, the solid black line indicates mean bird-lure trap CPUE and dashed line is mean mouse-lure trap CPUE from this study.

Discussion

Traps with live bird lures had a contact rate with snakes that was almost 15 times greater than the number of snakes that were successfully captured. Unpublished data from cameras referred to in Clark et al. (2012) and visual observations using night-vision goggles (G. Rodda, personal communication) at mouse-lure traps also showed that most snakes that tried to enter the traps failed to do so. Such collective evidence suggest that such trapping failures are common and that some snake individuals may be more difficult to trap. Despite the fact that 87% of our bird-lure traps failed to capture a snake, prior research in a 5 ha enclosed area of snakes has shown that all snakes of trappable size can be trapped in mouse-lure traps given intensive trapping over time (Tyrrell et al. 2009). Brown treesnakes begin to prey on birds around 750–950 mm SVL (Siers 2015), similar to the size at which they become trappable using live mouse lures (Tyrrell et al. 2009); of 555 snakes from various habitats with prey in their stomachs, the smallest BTS to contain an avian prey item (domestic fowl chick) was 717 mm SVL (Siers pers. comm.). Thus, with intensive effort, mouse-lure traps can target individual snakes large enough to consume birds. However, our results suggest that a trapping effort that is less than landscape-scale saturation (e.g., our trapping grid of 7.65 ha out of 51 ha behind a barrier) removed only 20% of snakes that entered a bird-lure trap and did not suppress snakes enough to reduce contact rates with birds.

Savidge (1991) noted that mouse-lure traps along the edge of a trapping grid captured more brown treesnakes than did traps deployed in interior portions of the grid. The difference in captures between our edge and interior traps was at least partially explained by high captures rates on the transect line (F) parallel to a small cliff-line ridge and abundant habitat. The overall increase in the number of brown treesnakes captured in traps placed at the edge of the grid suggests brown treesnake depletion was not occurring at least in part due to continued immigration into the trapping grid. Effective barriers (Rodda et al. 2007) adjacent to control areas can eliminate snake immigration (Tyrrell et al. 2009; Christy et al. 2010), but our trapping grid was not immediately adjacent to the snake-proof barrier. Snake density associated with the trapping grid is unknown, so we are unable to determine if reduced interior contact rates for mouse-lure traps resulted from an overall reduction in brown treesnakes or reduced interest in mouse-lure traps from those snakes that remained. The fact that shifting bird-lure traps had equivalent contact rates prior to snake removal as they did afterwards indicates that the stationary mouse-lure traps did not adequately reduce the density of snakes interested in birds.

Even though the landscape around the bird-lure traps had a high density of mouse-lure traps, most (68%) of the snakes that were captured in bird-lure traps were not recaptured in either bird- or mouse-lure traps. Mouse-lure traps, however, captured more snakes per unit effort than bird-lure traps, a finding documented in another study at the GNWR (Klug et al. 2015). Mice may produce a generally more attractive or stronger odor plume than birds (Rodda et al. 1999). Alternatively, the strong edge effect on trap capture success combined with the fact that bird-lure traps were always deployed in the grid interior may have partially driven the different capture success documented in this study. Additionally, snakes that entered bird-lure traps tended to be larger and in better body condition. Quail (150–180 g) are much larger than mice (20–40 g) and if brown treesnake size partially drives prey preference (Savidge 1988), then quail may be attractive to slightly larger snakes. Overall, large snakes are less common on Guam except in urban locations (Savidge 1991; Siers et al. 2017) and thus there may be few snakes on GNWR that are large enough to be attracted to quail.

While quail may be too large for many snakes, mice should still be of interest to larger snakes, as rodents are an important component of the diet of snakes >800 mm SVL on Guam (Savidge 1988; Siers 2015). Despite this, only 20% of all snakes captured in bird-lure traps were ever captured in stationary mouse-lure traps, despite the abundance of nearby mouse-lure traps in Phases II and III. The recapture rate for brown treesnakes captured in a bird-lure trap was 32% overall, which suggests that these snakes did not fully avoid traps. Whether a morphological- or individual-based preference, our observation that some snakes were willing to enter a trap with a bird but not a mouse lure provides limited evidence that snakes may vary in their dietary preferences. Many animals have been documented to specialize on a small subset of the dietary breadth of their species (Bolnick et al. 2002a, 2002b). We do not think that the stationary nature of mouse-lure traps combined with the weekly shifting of bird-lure locations is problematic for our interpretations because we maintained both spatial and temporal balance of traps and lure types throughout our study period.

Populations can experience trait changes in response to harvesting pressure (Palkovacs et al. 2018). Invasive species control measures resulting in non-random removal of individuals from targeted populations can lead to population-level shifts in mean trait values (Zavorka et al. 2018). Selection that reduces control tool efficacy within a population can be minimized by implementing multiple tools/lures to remove individuals from the population, in hopes that different tools will target individuals with distinct trait values (Palkovacs et al. 2018). Therefore, multi-faceted control techniques that include alternate lure forms or distinct treatments occurring concurrently may improve the overall outcome of brown treesnake control to support bird recovery (as can multi-faceted control efforts for invasive rats; Russell et al. 2008).

Beyond the benefits of reducing individuals resistant to capture, a multi-faceted control approach is expected to improve efficacy for other reasons. For example, camera trap imagery demonstrated that snakes were highly motivated to contact birds, with one snake spending over 2 hours attempting to access the bird. To enter a trap, however, snakes must find the trap entrance. Thus, control techniques that require less problem-solving by the snakes (e.g., open-ended bait tubes) (Lardner et al. 2013; Clark et al. 2018) may increase the odds of successful bird reintroductions via enhanced snake control. Brown treesnakes have been dramatically suppressed in experimental test plots by aerial delivery of dead neonatal mouse baits treated with 80 mg of acetaminophen (Dorr et al. 2016; Siers et al. 2019). Use of live-lure trapping and aerial delivery of toxicants may target a higher proportion of the snake population by targeting snakes attracted to rodents but unable to easily solve the problem of how to enter a trap. In contrast, individuals that are attracted to live prey over carrion may be more effectively targeted by traps with live lures. There is some evidence that carrion is less attractive to very large brown treesnakes (Shivik et al. 1999) and thus live-lure traps may be an essential component for targeting the largest individuals in a population.

Remote cameras aimed at bird-lures reliably captured nocturnal brown treesnake presence and behavior but required the use of high frequency photography (30-second intervals) because snakes failed to trigger the infrared sensors. Of the other potential nest predators detected (feral pigs, rats, monitor lizards, and cats), the high nocturnal sighting rates of feral pigs (1.43 pigs/camera trap night) would be problematic for reintroduced ground-nesting birds (e.g., Guam rail; *Gallirallus owstoni*). Cameras in association with avian lures may have a promising role in assessing predation risk or may act as a sentinel for detecting snake ingress into previously snake-eradicated areas. We recognize that all successful lures used in snake control to date rely on a food attractant (Rodda et al. 1999) and that snake suppression will allow recovery of prey populations, thus depressing future snake detection via food-based lures. That said, we documented that 44% of the snakes viewed on cameras failed to physically interact with the trap and its lure (Fig. 3).

The average interval between snake captures in bird-lure traps also decreased with time (from 10.4 days during the first 5-week interval to 1.6 days during the last 5-week interval), suggesting that the longer traps were on the landscape the more frequently they were visited by snakes. Odor cues from the traps may have accumulated, attracting snakes from greater distances. It is also possible that snakes were drawn in gradually at a constant rate (either from the scent or random movements in the landscape), without any increase in the grid's attraction rate, and that bird-attracted snakes (not removed by mouse-traps in Phase III) simply became increasingly common as they decided to move no further but to stay near birds. Alternatively, the study progressed in time through the wet season and trapping during the wet season has been shown to result in higher CPUE (Nafus et al. 2018).

Conclusions

Snake trapping around a small-scale simulated bird reintroduction site (bird-lure traps) did not demonstrably reduce brown treesnake contact rates with birds as compared to trap-contact rates prior to initiating snake removal in a snake-suppressed landscape. Trapping efforts required to meaningfully suppress brown treesnakes in support of bird recovery over large areas of Guam are assumed to be cost-prohibitive. Integration of new technologies such as the aerial delivery of toxicants is likely to be required to sufficiently suppress snakes at spatial scales large enough to support bird restoration efforts. However, this study provides evidence that some snakes may select live birds over live mouse lures, and thus reliance on a single control tool and lure may be inadequate for support of avian reintroductions and could lead to unintended harvest-driven trait changes within snake populations. Integration of multiple control tools and multiple lures is thus thought to yield the best management outcomes for reintroduction and recovery of native vertebrate species on Guam.

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Supplementary material 1

Select camera images of a failed brown treesnake (Boiga irregularis) trap capture using a bird lure

Authors: Amy A. Yackel Adams

Data type: TIF File (.tif)

Explanation note: Select time-lapse camera photos (4 images) of a failed capture at a bird-lure trap by a single brown treesnake. This individual attempted to secure the bait for 35 minutes and 30 seconds before leaving the trap area. White arrows point to the eye shine of the snake. Overall, camera traps revealed a much higher snake contact rate with bird lures than did bird-lure live trap data alone.

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RESEARCH ARTICLE

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The potential evolutionary impact of invasive balloon vines on native soapberry bugs in South Africa

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Abstract

Following their establishment in new communities, invasive species may cause evolutionary changes in resident native species. This is clearly true for phytophagous insects, which may adapt rapidly when utilising abundant and widespread introduced hosts. The balloon vines *Cardiospermum halicacabum* and *C. grandiflorum* were introduced to South Africa approximately 100 years ago and are classified as minor and major weeds, respectively. Here we assess the potential evolutionary impact of these vines on native *Leptocoris* soapberry bug populations in Kruger National Park (KNP), using phylogenetic and morphometric analyses. We found that soapberry bugs associated with *C. halicacabum* are genetically and morphologically distinct from those associated with *C. grandiflorum*. This suggests that native soapberry bugs in KNP exhibit some degree of host preference, indicating that these vines may have had significant evolutionary consequences for these insects. The proboscis length of soapberry bugs feeding on *C. halicacabum* closely matched fruit size, often being longer than fruit size at the population level. These soapberry bugs are therefore well-suited to feeding on this introduced plant species.

Keywords

Balloon vine, *Cardiospermum*, invasive species, Kruger National Park, *Leptocoris*, rapid evolution, soapberry bug

Introduction

Evolved and plastic changes are important not only for the colonization and spread of non-native species but are also prevalent in native species as they respond to the presence of introduced taxa (Mooney and Cleland 2001; Strauss et al. 2006; Ghalambor et al. 2007). Common examples of rapid evolutionary change in native species in response to non-native species come from herbivores, particularly phytophagous insects, adapting to introduced plants as preferred hosts (Carroll 2007). For example, evidence for this comes from the study of soapberry bugs (Hemiptera: Rhopalidae), which are specialised predators of seed from the Sapindaceae family (Carroll and Loye 1987). Soapberry bugs feed on the seeds of Sapindaceae species using their elongated, needle-like proboscides to pierce fruits and reach the seeds they contain (Carroll and Loye 1987; Carroll and Boyd 1992). Native soapberry bugs in North America and Australia have colonised several introduced Sapindaceae species (Carroll and Loye 2012). These bugs have subsequently undergone significant adaptive changes in the length of their proboscides and other allometries, directly related to the fruit size of the introduced hosts they utilise (Carroll and Boyd 1992; Carroll et al. 2005a). These changes are heritable and adaptive, facilitating greater feeding efficiency and reproductive success on their new hosts (Carroll et al. 2005a) and have occurred over just 20–50 years (about 40–150 bug generations), indicating the significant selection pressure exerted by these introduced hosts on their newly acquired soapberry predators (Carroll and Boyd 1992; Carroll et al. 2005a). Ultimately, such evolutionary responses in native species could alter contemporary community dynamics and the assembly of future communities, even leading to incipient speciation between native subpopulations (Strauss et al. 2006; Carroll 2007; Andres et al. 2013). Importantly for biological invasions, the rapid evolution of native taxa in response to invaders may impede an invader's spread (Carroll 2011).

The genus *Cardiospermum* L. (Sapindaceae), commonly known as balloon vines, consists of 17 mainly Neotropical species (Gildenhuys et al. 2013). Three species, *C. grandiflorum* Sw., *C. halicacabum* L., and *C. corindum* L., have near-cosmopolitan distributions, although their provenance as native or introduced species remains unresolved in some regions (Gildenhuys et al. 2013). Gildenhuys et al. (2014) showed that *C. corindum* is native to both southern Africa and South America, and confirmed that *C. grandiflorum* and *C. halicacabum* are modern arrivals in southern Africa. *Cardiospermum* species are considered invasive in many parts of the world, often as an unplanned result of intentional introductions for ornamental and medicinal purposes (Carroll et al. 2005b; Simelane et al. 2011; Gildenhuys et al. 2013). Invasive *Cardiospermum* species are commonly classified as 'transformer' weeds, as they often cover native vegetation, potentially driving local biodiversity loss (Henderson 2001; Mc Kay et al. 2010). *Cardiospermum grandiflorum* and *C. halicacabum* were introduced to South Africa approximately 100 years ago (Simelane et al. 2011; Gildenhuys et al. 2013) and are currently listed as Category 1b and Category 3 invaders, respectively, under the National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004). Category 1b species 'may not be owned, imported into South Africa, grown, moved, sold, given as a gift or dumped in a waterway'. These species are major invaders requiring containment and removal, often with the assistance of government sponsored programs (Alien and Invasive Species Regulations 2014; https://www.environment.gov.za). Category 3 species may remain in certain areas/provinces, but further propagation or trade is prohibited. In South Africa, *C. grandiflorum* is found along most of the Kwa-Zulu Natal coast and in the Gauteng, Limpopo and Mpumalanga provinces (Henderson 2001). *Cardiospermum halicacabum* is less widely distributed in South Africa, largely restricted to the Limpopo and Mpumalanga provinces (Henderson 2001). The high level of specialisation and co-occurrence of soapberry bugs with *Cardiospermum* suggests that soapberry bugs may play an important role in regulating the reproduction of *Cardiospermum* globally (Gildenhuys et al. 2013). For example, seed predation levels by soapberry bugs on native *C. corindum* in Florida can be as high as 90 % (Carroll 1988; Carroll et al. 2003).

Two soapberry bug genera are native to southern Africa, namely *Leptocoris* and *Boisea*, the former being more widely distributed (Göllner-Scheiding 1980, 1997; Carroll and Loye 2012). Twenty-one *Leptocoris* species are endemic to Africa, in association with native Paullinieae and Thouinieae hosts (Carroll and Loye 2012). In South Africa, native *Allophylus* species are common hosts, but native soapberry bugs have also colonised *C. grandiflorum* and *C. halicacabum* in many parts of the country (Carroll and Loye 2012; JF *pers. obs.*). The *Cardiospermum*-soapberry bug system is well-studied in North America and Australia but remains little-explored in the South African context.

In this study, we used phylogenetic analyses, in combination with morphometric measurements, to investigate the potential evolutionary impact of invasive *C. halicacabum* and *C. grandiflorum* on native soapberry bugs (genus *Leptocoris*) in South Africa. To examine whether any evolved differentiation might have resulted from contemporary natural selection, the proboscis lengths of *Leptocoris mutilatus* Gers. were measured with respect to fruit size variation in invasive *C. halicacabum* populations. The expectation was that soapberry bug proboscis lengths will closely match fruit size in *C. halicacabum* and that any shifts in proboscis lengths will correspond to variation in seed capsule (balloon) size in *C. halicacabum* populations.

Methods

Sampling

Thirteen populations of *C. halicacabum* and a single *C. grandiflorum* population were identified in Kruger National Park (KNP; Suppl. material 1: Table S1). Only one population of *C. grandiflorum* was surveyed as local eradication programmes made it difficult to locate more populations in KNP. *Cardiospermum halicacabum* populations were approximately one kilometre apart and the *C. grandiflorum* population was 21.5 km from the nearest *C. halicacabum* population. Adult soapberry bugs present on individuals in these populations were collected and preserved in 70% ethanol. The initial field classification of all collected soapberry bugs was *Leptocoris mutilatus*.

Leptocoris mutilatus is a typical soapberry bug, about 11–16 mm in length, characterised by an overall scarlet red or brownish red colour and a black, bulged head (Göllner-Scheiding 1980). The species has a wide native range distribution, including Madagascar and central, eastern and southern Africa (Göllner-Scheiding 1980).

***Leptocoris* phylogeny**

To confirm the putative *Leptocoris mutilatus* species assignment of soapberry bugs feeding on invasive *Cardiospermum* in KNP, individuals collected from *C. halicacabum* and *C. grandiflorum* populations were selected for phylogenetic analyses (Suppl. material 1: Table S2). An individual bug from five *C. halicacabum* populations and five individuals from the *C. grandiflorum* population were included in these analyses (Suppl. material 1: Table S2). For those bugs associated with *C. halicacabum*, individuals were selected from populations at different distances from one another to minimise potential isolation by distance effects. Reference specimens of six African species and a single Asian *Leptocoris* species were included in the phylogenetic analyses (Suppl. material 1: Table S2). Bug specimens were preserved in 70% ethanol at -80 °C. Genomic DNA was extracted from legs or whole bodies using the DNeasy Blood and Tissue Kit (Qiagen, supplied by WhiteHead Scientific, Cape Town, South Africa) following the manufacturer's instructions. DNA quality and quantity were assessed using NanoDrop ND-1000.

The mitochondrial cytochrome c oxidase subunit I (*COI*) gene was amplified using the universal primers LCOI-1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCOI-2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994). Each 30µl reaction contained approximately 100 ng of genomic DNA, 0.2 mM of each dNTP (Fermentas, Thermo Fisher Scientific, Waltham, Massachusetts, United States), 0.5 µM of each primer, 1 U Taq DNA polymerase [Supertherm, Separation Scientific SA (Pty) Ltd, Roodepoort, South Africa], 1 × PCR reaction buffer, 2 mM MgCl2 and 0.2 mg/ml BSA (Promega). PCR cycles consisted of initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and elongation at 72 °C for 1 min, and final extension at 72 °C for 30 min.

All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, supplied by Whitehead Scientific, Cape Town, South Africa). Purified products were sequenced using an ABI 3730 XL automated machine (Central Analytical Facilities, Stellenbosch University, South Africa).

DNA sequence data were aligned using CLUSTALW version 2.1 (Thompson et al. 2003), followed by manual editing in BIOEDIT version 7.0.5.3 (Hall 1999). The final *COI* dataset consisted of 26 accessions: 10 putative *L. mutilatus* from KNP (five from *C. halicacabum* and five from *C. grandiflorum*), two reference specimens of *L. mutilatus*, two putative *L. amictus*, seven putative *L. hexophtalmus*, one *L. productus*, two *L. aethiops* and two *L. vicinus* (Suppl. material 1: Table S2). Two outgroup sequences of *Boisea trivittata* (JX629056.1 and JX629057.1), a species in the same subfamily as *Leptocoris*

(Serinethinae), were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and used as outgroup taxa. Species identifications were performed with reference to Göllner-Scheiding (1980, 1997).

A phylogeny was reconstructed using Bayesian inference (BI) and maximum likelihood (ML) approaches. Bayesian inference was conducted using MRBAYES version 3.2.6 (Ronquist et al. 2012) through the CIPRES Science Gateway version 3.3 (Miller et al. 2010). The best-fit DNA substitution model was identified in JMODELTEST version 2.1.6 (Darriba et al. 2012) using Akaike's information criterion (AIC) (Akaike 1974). The chosen DNA substitution model for the *COI* locus was TPM2uf + I + Γ (-1nL = 1816.41, AIC = 6021.58) (Akaike 1974). The base frequencies were as follows, A = 30.10 %, C = 16.22 %, G = 14.56 % and T = 39.12 %. The rate matrix was R(a) [A-C] = R(c) [A-T] = 1.63, R(b) [A-G] = R(e) [C-T] = 10.96, R(d) [C-G] = R(f) [G-T] = 1.00, and the proportion of invariable sites (I) was 0.6540, and the gamma shaped distribution (Γ) was 1.6170. Each model was run for 10 million generations, sampling every 1000 generations using the default parameters. Consensus trees were built after discarding 20% of trees as burn-in and posterior probabilities (*PP*) were estimated based on the percentage of time spent on node recovery. Posterior probability values of < 0.90 were regarded as poor support.

Maximum likelihood analysis was conducted using GARLI version 2.01 (Zwickl 2006) through the CIPRES Science Gateway version 3.3 (Miller et al. 2010). Bootstrap analysis was used to generate branch support values (1000 pseudo-replicates) (Felsenstein 1985). Bootstrap values of < 75% were regarded as indicating poor support. Tree search analysis was performed using a heuristic search algorithm starting at a random tree. Base frequencies were estimated, and four rate categories were included for gamma. Final bootstrap values were discerned via a 50% majority rule tree, generated using PAUP version 4.0 (Swofford 2002).

Allometry of native *Leptocoris* and trait-matching with invasive *Cardiospermum* populations

To investigate whether soapberry bug proboscis lengths track fruit size variation in *C. halicacabum* populations, a total of 311 full-sized fruit and 154 associated soapberry bug individuals were measured from 13 *C. halicacabum* populations in KNP. 20–25 fruit and 5–16 soapberry bugs were measured for each population (Suppl. material 1: Table S3). To compare allometries between bugs found on *C. halicacabum* and those found on *C. grandiflorum*, a further 30 soapberry bug individuals from the single *C. grandiflorum* population were measured. Following Carroll and Loye (1987), the proboscis length of each bug was measured from the anterior tip of the clypeus to the distal tip of the proboscis. Thorax width and body length, taken from the anterior tip of the clypeus to the distal tip of the abdomen, were measured as proxies for body size. For the fruit measurements a cross-section was made just above the seeds using a pair of sharp scissors (Figure 1). Extra care was taken not to deform or compromise

Figure 1. Interior of fruiting capsule of balloon vine *C. halicacabum*. The upper portion of the capsule wall has been trimmed away and shows the central position of the seeds. The 'fruit size' variable we measured was the shortest distance from the fruit capsule perimeter to the seed coat for each seed. The second species in this study, *Cardiospermum grandiflorum*, has larger fruits of similar architecture (Image source: Wikimedia commons: *Cardiospermum halicacabum*).

the integrity of the membranes holding the seeds in place. Fruit size was measured as the shortest distance from the fruit capsule perimeter to the seed coat and repeated for every seed inside each fruit. A further 14 fruits were measured from the single *C. grandiflorum* population in KNP. Bug and fruit measurements were taken using handheld digital callipers with a 0.01 mm resolution.

ANCOVA models were used to quantify and compare proboscis-body size allometries between bugs found on different *Cardiospermum* hosts. For those bugs found on *C. halicacabum*, regression analysis was used to determine whether there was a significant trait-matching relationship between mean population proboscis lengths and mean population fruit sizes. A two-sample *t*-test was used to determine whether mean proboscis length was significantly different between bugs found on *C. grandiflorum* and *C. halicacabum*. A Kruskal-Wallis rank sum test and post-hoc Dunn's test were used to compared proboscis lengths at the population level. Two-sample *t*-tests were also used to determine whether fruit size was significantly different between balloon vine species. Furthermore, two-sample *t*-tests were used to determine the nature and direction of the morphological fit between the fruit sizes of each *Cardiospermum* host and the proboscis lengths of their associated soapberry bug predators.

All statistical analyses were conducted in R (R Core Team 2018).

Results

Sequence variation

The aligned *COI* dataset contained 545 base pairs. All sequences were deposited into the GenBank online repository.

Leptocoris phylogeny

The phylogeny recovered well-supported basal clades for *Leptocoris vicinus* and *L. aethiops*, and a larger clade containing *L. amictus* and *L. hexophtalmus* (Figure 2). All KNP soapberry bugs fell into a monophyletic clade with the representative *L. mutilatus* specimens, sister to *L. productus* ($PP = 0.99$; Figure 2). However, while *Leptocoris* specimens collected from *C. grandiflorum* in KNP grouped unequivocally with *L. mutilatus* reference specimens, all the soapberry bugs collected from *C. halicacabum* fell into a single clade ($PP = 0.99$) sister to the clade containing specimens from *C. grandiflorum* and *L. mutilatus* reference specimens (Figure 2). This suggests that while those bugs feeding on *C. halicacabum* are likely *L. mutilatus*, they are genetically differentiated from those feeding on *C. grandiflorum*. Based on this differentiation, soapberry bugs found on *C. halicacabum* in KNP will hereafter be referred to as 'halicacabum bugs' and those on *C. grandiflorum* as 'grandiflorum bugs'. The ML analysis produced a near-identical tree topology to the BI analysis, but nodal support was generally weaker (Figure 2).

Allometry of native *Leptocoris mutilatus* and trait-matching with invasive *Cardiospermum* populations

There was a significant positive relationship between proboscis length and thorax width for both halicacabum ($p < 0.001$; Table 1) and grandiflorum bugs ($p < 0.01$; Table 1; Figure 3). Likewise, there was a significant positive relationship between proboscis length and body length in halicacabum bugs ($p < 0.001$; Table 1; Figure 4). No significant relationship was found between proboscis length and body length in grandiflorum bugs ($p > 0.05$; Figure 4). Halicacabum bugs had a significantly stronger relationship between proboscis length and thorax width than grandiflorum bugs ($F_{1,180} = 17.03$, $p < 0.001$; Table 1; Figure 3). Similarly, halicacabum bugs had a significantly stronger relationship between proboscis length and body length than grandiflorum bugs ($F_{1,180} = 15.07$, $p < 0.001$; Table 1; Figure 4). However, at the population level, only six halicacabum bug populations showed a significantly stronger relationship for both the proboscis-thorax width and proboscis-body length allometry compared to the grandiflorum bug population ($p < 0.05$; Suppl. material 1: Table S4). Three halicacabum bug populations did not have significantly stronger relationships for both the proboscis-thorax width and proboscis-body length allometry compared to the grandiflorum bug population ($p >$

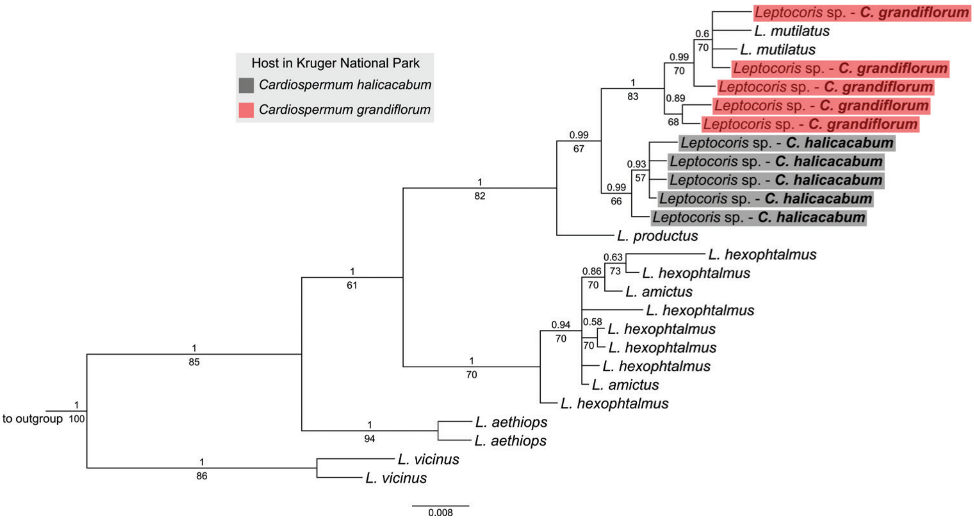

Figure 2. Bayesian phylogeny based on *COI* DNA sequencing data illustrating phylogenetic relationships among African *Leptocoris* species. Shaded branches refer to *Leptocoris* specimens collected from *Cardiospermum halicacabum* and *C. grandiflorum* in Kruger National Park. Nodal support is shown as posterior probabilities and bootstrap values above and below the branches, respectively.

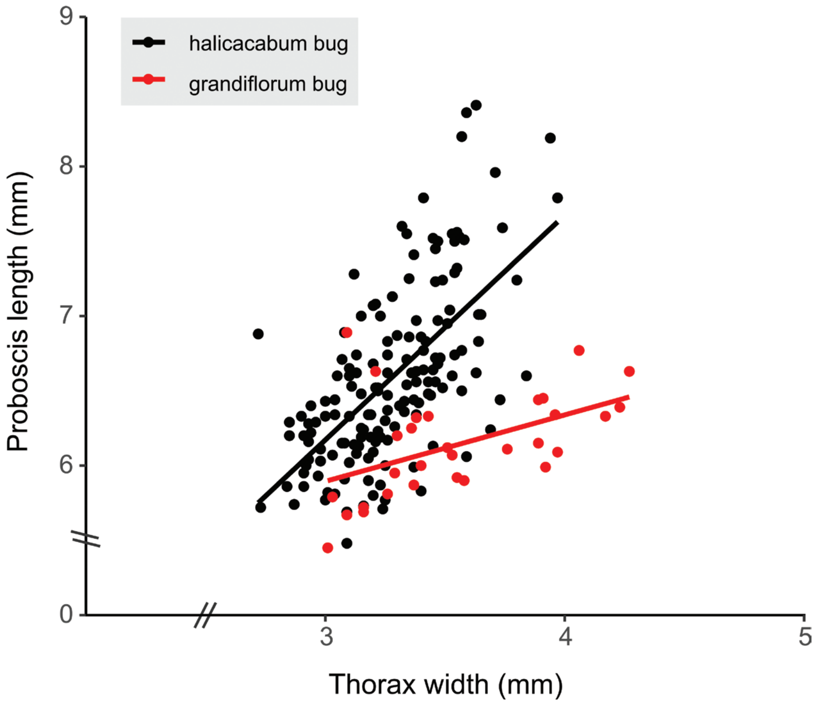

Figure 3. Linear regressions showing the relationship between proboscis length and thorax width for adult *Leptocoris mutilatus* found in association with *Cardiospermum halicacabum* and *C. grandiflorum*. Each point represents an individual.

Table 1. Details of the models used to quantify and compare allometries of soapberry bugs in KNP, and to assess the relationship between population means of halicacabum bug proboscis length (in mm) and *C. halicacabum* fruit size (in mm).

LM (Proboscis length ~ Thorax width * Host plant)				
	Estimate	*SE*	*t*-value	*p*-value
(Intercept)	1.6628	0.4718	3.524	< 0.001
Thorax width	1.5029	0.1436	10.467	< 0.001
Host *C. grandiflorum*	2.8979	0.8949	3.238	< 0.01
Thorax width: Host *C. grandiflorum*	-1.0584	0.2565	-4.126	< 0.001
Residual *SE*: 0.439 on 180 df				
Multiple r^2: 0.4371, Adjusted r^2: 0.4277				
F-statistic: 46.59 on 3 and 180 df, *p*-value: < 0.001				
LM (Proboscis length ~ Body length * Host plant)				
	Estimate	*SE*	*t*-value	*p*-value
(Intercept)	2.1152	0.5319	3.977	< 0.001
Body length	0.3799	0.0451	8.431	< 0.001
Host *C. grandiflorum*	3.0109	0.9499	3.170	< 0.01
Body length: Host *C. grandiflorum*	-0.2989	0.0770	-3.883	< 0.001
Residual *SE*: 0.4734 on 180 DF				
Multiple r^2: 0.3454, Adjusted r^2: 0.3345				
F-statistic: 31.66 on 3 and 180 df, *p*-value: < 0.001				
LM (Mean proboscis length ~ Mean fruit size)				
	Estimate	*SE*	*t*-value	*p*-value
(Intercept)	6.8366	0.7885	8.671	< 0.001
Mean fruit size	-0.0411	0.1262	-0.326	> 0.05
Residual *SE*: 0.1832 on 11 DF				
Multiple r^2: 0.0095, Adjusted r^2: -0.0805				
F-statistic: 0.1062 on 1 and 11 df, *p*-value: > 0.05				

0.05; Suppl. material 1: Table S4). Four halicacabum bug populations had significantly different relationships for one allometry but not the other compared to the grandiflorum bug population (Suppl. material 1: Table S4). The coefficient of variation for proboscis length was 8.95% in halicacabum bugs and 5.59 % in grandiflorum bugs.

There was no significant relationship between population means of *C. halicacabum* fruit size and proboscis lengths of their associated soapberry bug predators ($p > 0.05$; Table 2; Figure 5). Furthermore, there was no significant relationship between population means of the halicacabum bug allometry residuals and *C. halicacabum* fruit size ($p > 0.05$; Suppl. material 1: Table S5). Mean halicacabum bug proboscis length was significantly longer than grandiflorum bugs (Student's *t*-test, $t = 4.00$, df = 182, $p < 0.001$; Table 2). Similarly, proboscis length was significantly different between halicacabum bugs and grandiflorum bugs at the population level (Kruskal-Wallis rank sum test, $\chi^2 = 29.09$, df = 13, $p < 0.01$). However, post-hoc analysis showed only one halicacabum bug population had significantly longer proboscides than grandiflorum bugs, namely population Hali7 (Dunn's test, $z = 3.63$; $p < 0.05$). Fruit size of *C. halicacabum* was significantly smaller than that of *C. grandiflorum* (Student's *t*-test, $t = 6.40$, df = 323, $p < 0.001$; Table 2). However, halicacabum bug proboscis length was significantly

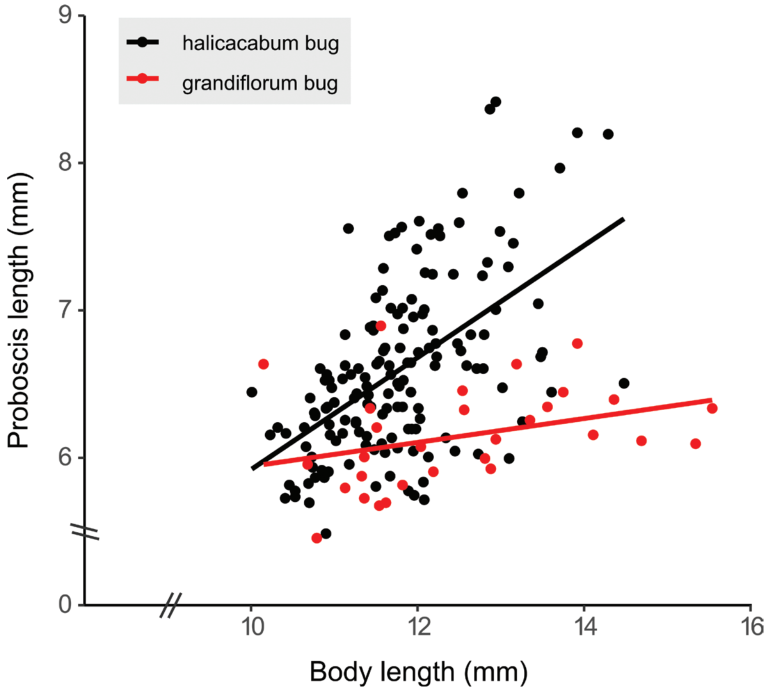

Figure 4. Linear regressions showing the relationship between proboscis length and body length for adult *Leptocoris mutilatus* found in association with *Cardiospermum halicacabum* and *C. grandiflorum*. Each point represents an individual.

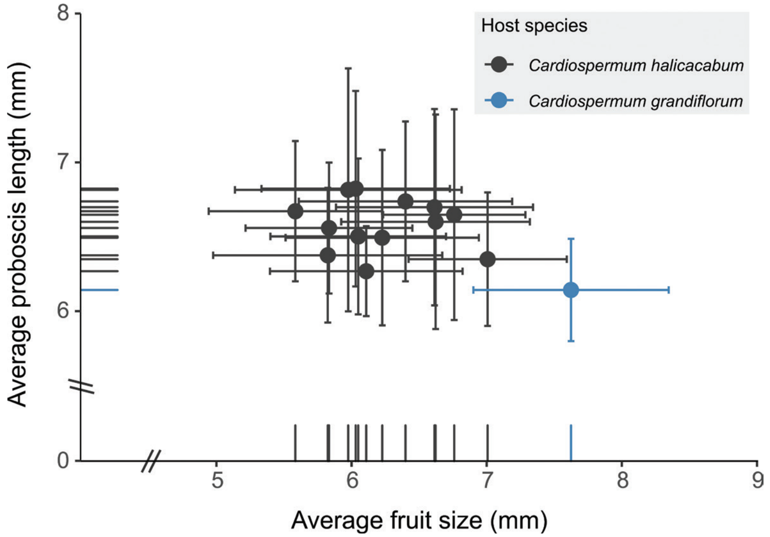

Figure 5. Relationship between proboscis length and fruit size (distance from balloon exterior to nearest seed coat) for soapberry bugs and their associated balloon vine host species in Kruger National Park. Each point represents a single population and error bars are ±SD.

Table 2. Grand means (±SD) of proboscis length and body size measures (in mm) of soapberry bugs in KNP, and fruit sizes (in mm) of their *Cardiospermum* hosts.

Host plant	Proboscis length (mm)	Thorax width (mm)	Body length (mm)	Fruit size (mm)
Cardiospermum halicacabum	6.59 ± 0.59	3.28 ± 0.25	11.77 ± 0.85	6.24 ± 0.80
Cardiospermum grandiflorum	6.14 ± 0.34	3.56 ± 0.38	12.54 ± 1.41	7.62 ± 0.72

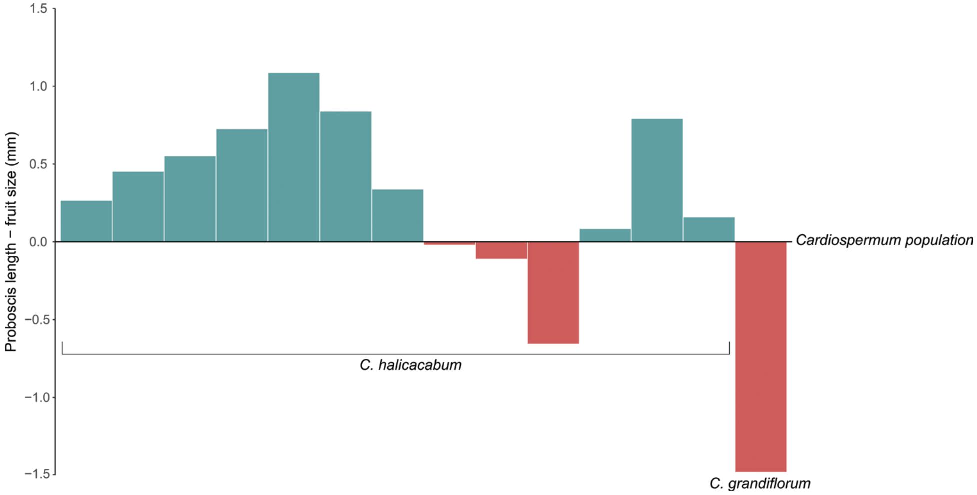

Figure 6. Difference between average proboscis length and average fruit size (i.e. proboscis length - fruit size) for *Leptocoris mutilatus* and *Cardiospermum* populations in KNP. Green bars represent populations in which soapberry bugs are well-suited to feed on their *Cardiospermum* hosts (i.e. proboscis length > fruit size) and red bars represent populations in which soapberry bugs are poorly matched (i.e. proboscis length < fruit size).

longer than the fruit size of their *C. halicacabum* hosts (Student's *t*-test, $t = 4.83$, df = 463, $p < 0.001$; Figure 6, Table 2). In contrast, grandiflorum bug proboscis length was significantly shorter than the fruit size of their *C. grandiflorum* hosts (Welch's *t*-test, $t = 7.29$, df = 15.81, $p < 0.001$; Figure 6, Table 2).

Discussion

This study aimed to assess the potential for impacts by invasive balloon vines on morphological traits of native soapberry bug (genus *Leptocoris*) populations in South Africa's flagship protected area, Kruger National Park (KNP). We found *L. mutilatus* bugs on both invasive host plants and provide some evidence for genetic and morphological differentiation between bugs feeding on different balloon vine species in KNP. Additionally, the proboscis-fruit size trait-matching patterns differed between the two balloon vines. Halicacabum bugs had proboscis lengths well-suited for feeding on the seeds of intact fruits, but this was not the case for grandiflorum bugs.

Genetic differentiation between soapberry bug populations associated with *C. halicacabum* and *C. grandiflorum* in KNP (Figure 2) was mirrored by morphological differentiation between these populations (Figures 3, 4). More specifically, halicacabum bugs had significantly longer proboscides and stronger proboscis length-body size allometries than grandiflorum bugs. This host-associated morphological differentiation agrees with Carroll and Boyd (1992), who found significantly different relationships between proboscis length and body length for another soapberry bug, *Jadera haematoloma*, utilising different native and introduced Sapindaceae species in North America. Similarly, Carroll et al. (2005a) found evidence for a breakdown of proboscis length-body size allometry in an Australian soapberry bug, *Leptocoris tagalicus*. The proboscis length of *L. tagalicus* feeding on introduced *C. grandiflorum* increased relatively more than body size in females and without concomitant body size changes in males compared to native host plant-feeding bugs (Carroll et al. 2005a). Importantly, cross-rearing experiments showed that these changes are heritable, with a strong signal of host-specific genetic differentiation (Carroll et al. 2005a). The moderate host-specific phylogenetic signal we retrieved may point to similar differentiation between halicacabum and grandiflorum bugs in South Africa, i.e. the incipient stages of partial reproductive isolation and therefore 'host race' formation. However, the sampling of a single grandiflorum bug population strongly restricts any inferences made here. In addition to the lack of significant morphological differentiation between halicacabum and grandiflorum bugs for all populations, the amount of variation observed between halicacabum populations suggests that further sampling of bugs from *C. grandiflorum* populations in KNP may reveal overlapping morphological variation between bugs on each host. Therefore, while the genetic and morphological evidence provides some support for incipient host race formation, our sampling bias does not allow us to conclusively reject the possibility that a single variable *L. mutilatus* lineage exploits both invasive balloon vine hosts in KNP. Furthermore, the halicacabum bug populations were geographically distant from the grandiflorum bug population (separated by 21.5 km), suggesting that the genetic differentiation between halicacabum and grandiflorum bugs may be partly explained by isolation by distance and not only host-specific genetic differentiation. Alternatively, the allometries of these soapberry bugs may not be driven by their respective novel hosts at all, but derive rather from distinct ancestral populations with inherently different allometries. Future work should include a wider geographic collection of numerous populations from both native and balloon vine host plants in order to gain an improved understanding of these possibilities.

The lack of a significant proboscis length-fruit size trait-matching relationship across halicacabum bug populations (Figure 5) is perhaps unsurprising considering the limited variation in fruit size between populations (i.e. there is no significant fruit size variation to select for different proboscis lengths in the associated soapberry bug predators). Yet, the match between proboscis length and fruit size is very close (0.35 mm difference in means, Table 2). Importantly, 77% of the halicacabum bug populations had proboscis lengths longer than the average fruit size of their host population (Figure 6). This provides strong evidence that these bugs are well-suited to efficiently feed on *C. halicacabum* in KNP. Soapberry bugs may therefore have played a role in impeding the spread of *C. halicacabum* in KNP. Future studies should aim to quantify seed predation of introduced Sapindaceae species by soapberry bugs to assess their potential as 'neoclassical' biocontrol (Carroll 2011).

While the fit between proboscis length and fruit size is likely adaptive (Carroll and Boyd 1992, Carroll et al. 2005a), the mechanism behind it should be considered within the plant community context. There is at least one common native sapindaceous species in the immediate study area with fruit sizes that potentially overlap with *C. halicacabum*, namely *Pappea capensis.* This species has large seeds held singly within fruits of 10–15 mm diameter (Palgrave 1992; LF *pers. obs.*) with the distance from the fruit exterior to the seed being ~5mm (SPC *pers. obs.*). In addition, native *Cardiospermum corindum* (Siebert et al. 2010; Gildenhuys et al. 2014) and *Allophylus* species have also been collected from the region (Victor and van Wyk 2005) and could potentially also be sources of colonists. Accordingly, while the fit of halicacabum bug proboscis lengths may result from local adaptation to that host, it could also represent evolution by spatial sorting of migrant genotypes (*sensu* Shine et al. 2011) from surrounding native sapindaceous species with proboscis lengths suitable for feeding on balloon vines. However, considering the small range of proboscis lengths (coefficient of variation = 8.95%) and evidence for introduced balloon vine host specificity by soapberry bugs in other parts of the world (Carroll et al. 2005a; Andres et al. 2013), it is plausible that the proboscis length of halicacabum bugs is under selection by *C. halicacabum* fruit size with allometries different from those feeding on native hosts. Further sampling of soapberry bugs from native sapindaceous hosts in the vicinity of our study area is needed to determine the extent of adaptation by halicacabum bugs.

Interestingly, the average proboscis length of grandiflorum bugs was 1.48 mm shorter than the average fruit size of the associated *C. grandiflorum* population, indicating that these bugs are less well-suited to feed on these fruits. This may imply that these bugs have recently colonised this balloon vine such that selection has not had long to act (Carroll et al. 2005a). It is also possible that no colonisers reaching this larger-fruited species, or their offspring, have had proboscides long enough to reach the encapsulated seeds (*sensu* Cenzer 2018). In that event longer beaks would not have been selected for. Such constrained evolution of access to *C. grandiflorum* seeds could then also help explain the greater relative proboscis lengths (steeper allometries) we observed in halicacabum bugs compared to grandiflorum bugs, which was unexpected considering the larger fruit of *C. grandiflorum*. Nonetheless, three halicacabum bug populations had similarly 'maladapted' proboscis lengths (Figure 6). Therefore, it is plausible that further sampling of grandiflorum bugs would reveal a comparable pattern of variable adaptation to inflated balloon vine seedpods in the region, and it is indeed intriguing that some halicacabum bugs have suitably long beaks to access seeds in some *C. grandiflorum* fruits (Figure 5). The ongoing KNP program to eradicate *C. grandiflorum* meant we did not find additional *C. grandiflorum* sites to survey bugs, and we likewise lack morphological data for soapberry bugs feeding on native sapindaceous species in the region. Hence it remains unclear whether halicacabum or grandiflorum bugs have the more 'ancestral' allometry or whether the relative change is greater in either host race.

Not knowing about the potentially illuminating influences of native Sapindaceae fruit size on the allometries of halicacabum and grandiflorum bugs is a key shortcoming in this study, and any ongoing local patterns of host-associated differentiation inferred here may be changed by plant eradication efforts. Despite these challenges, our findings add support to considerations of the potentially significant evolutionary impact of introduced balloon vines on native soapberry bug populations, and that these impacts may be dissimilar for different balloon vine species. More extensive sampling in South Africa of soapberry bugs from both introduced balloon vine species and native sapindaceous species is needed to determine the degree of morphological and genetic differentiation between invasive- and native-feeding soapberry bug populations. This will provide a more complete assessment of the potential evolutionary impact of introduced balloon vines on soapberry bug populations, and the potential for these bugs as neoclassical biocontrol of *Cardiospermum* invasions.

Acknowledgments

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Supplementary material 1

Tables S1–S5.

Authors: Jarryd D. Foster, Allan G. Ellis, Llewellyn C. Foxcroft, Scott P. Carroll, Johannes J. Le Roux

Data type: measurements

Explanation note: **Table S1.** Sampling localities for *Cardiospermum* populations included in this study. **Table S2.** Locality data for *Leptocoris* species included in this study. **Table S3.** Number of fruit and soapberry bugs measured per population. **Table S4.** Population-level comparisons of proboscis length-body size allometries between halicacabum and grandiflorum bugs using ANCOVA models. **Table S5.** Details of the linear models used to assess the relationship between population means of the halicacabum bug allometry residuals (in mm) and *C. halicacabum* fruit size (in mm).

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Advancing research on alien species and biological invasions

Alien aquatic plants in Slovakia over 130 years: historical overview, current distribution and future perspectives

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Abstract

Alien aquatic plants rank amongst the major threats to aquatic biodiversity and, since ongoing climate change is expected to facilitate their further spread, there is an urgent need for sound knowledge of their distribution and ecology. We collected published and unpublished data spanning the last ~130 years and performed the first comprehensive assessment of alien aquatic vascular plants in Slovakia with the following aims: (i) to prepare a national inventory, (ii) to assess the effects of climate and landscape on species diversity and (iii) to evaluate the habitat preferences of the species. The historical overview showed a strongly increasing trend in the number of alien species related to an increased amount of intensive research of aquatic vegetation over the last 30 years. Altogether, 20 neophyte alien aquatic plant taxa were recorded from 479 sampling sites. However, the species inventory seems to be far from complete and approximately 14 species are expected to remain undetected. *Elodea canadensis* and *E. nuttallii* are the most frequently occurring alien aquatic plants, while eight other species have been found at a single site only. The majority of alien plants were deliberately introduced as aquarium ornamentals or released through pond waste. The fragmented information on local habitat conditions did not allow us to draw firm conclusions about the habitat preferences of alien aquatic plants. However, artificial water bodies are more frequently colonised by alien species than natural habitats (95% of aliens were found in artificial water bodies and 60% of them

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were recorded exclusively in these habitats) and many species have broad environmental tolerances (ability to colonise both standing and running waters, tolerances to a wide range of temperatures and water chemistry). Our results reaffirm the major role of increased temperatures and landscape modification in the distribution of alien aquatic plants and we can expect enhanced invasiveness and spreading of alien species into new habitats driven by climate change and land use intensification. Filling a main gap in the recognition of alien aquatic plant environmental preferences is a challenge for future research with the ultimate goal of maintaining natural aquatic plant diversity and ecosystem functioning.

Keywords

invasive species, macrophytes, aquatic weeds, distribution, climate change, land use

Introduction

Biological invasions by alien plants are generally recognised as an important component of human-induced environmental changes and they have a direct effect on the species diversity of various habitats (Manchester and Bullock 2000; Hulme 2003). Although water bodies have a relatively low level of invasion in Europe (Chytrý et al. 2009), these freshwater habitats are substantially influenced by alien plant species. Currently, almost 100 alien aquatic plants are recognised in Europe. However, the distribution pattern of this species in Europe is uneven; western, northern and central European countries, such as France, Italy, Germany or Hungary, are the most invaded, while some south-eastern European countries have a relatively low number of alien plants (Hussner 2012).

According to the Propagule, Abiotic, Biotic (PAB) framework (Catford et al. 2009), propagule pressure (e.g. the number of introduced individuals, seeds or propagules), abiotic (e.g. climatic or soil characteristics) and biotic (mutual relationships amongst species) variables are generally considered reasons for the presence, survival and success of alien species (Colangelo et al. 2017). While climate is important in setting the global range of alien species, factors related to human influence are of greater importance at regional scale (Kelly et al. 2014). Especially in the case of aquatic plants, trade and cultivation of aquatic ornamental plants are considered to be the main introduction routes for alien species (Duggan 2010; Hussner 2012). Moreover, the presence of alien aquatic species is usually positively correlated with shipping activity, tourism and human population size (Leuven et al. 2009; Panov et al. 2009; Hussner et al. 2010; O'Malia et al. 2018). Nunes et al. (2015) found that geographical patterns are related to some pathways of introduction of freshwater alien organisms in Europe: introductions through inland canals were concentrated in Central/North-eastern Europe, while introductions through pet/terrarium/aquarium trade were mainly observed in Central/Western Europe. In addition, thermal waters are key habitats for the establishment and survival of many alien aquatic plants. For example, approximately 80% of all detected non-indigenous aquatic plants in Hungary were found in thermal waters (Lukács et al. 2016).

Hussner (2012) reported only 6 alien aquatic plant species, namely, *Azolla filiculoides* Lam., *Crassula helmsii* (Kirk) Cockayne, *Elodea canadensis* Michx., *E. nuttallii* (Planch.) H. St. John, *Lemna minuta* Kunth and *Shinnersia rivularis* (A. Gray) R. M.

King & H. Rob. from Slovakia. The list was incomplete and information about the occurrence of *C. helmsii* was probably incorrect (Medvecká et al. 2012, http://dass.sav.sk/en/). Lukács et al. (2016) identified 48 alien aquatic plants from the Pannonian ecoregion including mainly Hungary and some parts of neighbouring countries, including Slovakia. The most recently published list of alien flora of Slovakia included the presence of 13 species (Medvecká et al. 2012). In addition to the species reported by Hussner (2012) and except for *C. helmsii*, eight additional species were included in the list (*Egeria densa* Planch., *Eichhornia crassipes* (Mart.) Solms, *Hydrilla verticillata* (L. f.) Royle, *Limnophila sessiliflora* Blume, *Najas guadalupensis* (Spreng.) Magnus, *Pistia stratiotes* L., *Sagittaria subulata* (L.) Buchenau and *Utricularia gibba* L.). Both sources pointed to a relatively low number of alien aquatic plants in freshwater habitats of Slovakia, which was also confirmed by a later study (Medvecká et al. 2014). During recent intensive limnological research, several new alien species were recorded and the volume of data on the distribution and ecology of alien aquatic plants in Slovakia increased substantially (e.g. Bubíková et al. 2016; Nobis et al. 2019). However, an exhaustive study on alien aquatic plants, their distribution and ecology was missing and the existing information remained scattered in various sources, many of them still unpublished.

Alien aquatic plants rank amongst the major threats to aquatic biodiversity (e.g. Strayer et al. 2010; Havel et al. 2015) and, since ongoing climate change is expected to facilitate the spread of these species (Lukács et al. 2016), there is an urgent need for sound knowledge of the distribution and ecology of alien aquatic species. Therefore, the aim of our study is to provide the first comprehensive examination of alien aquatic vascular plants in Slovakia, based on a critical review of all available data sources (published and unpublished). Our specific aims were to (i) prepare a national inventory of alien aquatic plants, (ii) assess the effect of climatic and landscape characteristics on alien species diversity and (iii) evaluate the habitat preferences of alien aquatic species. Subsequently, we discuss further trends in the distribution of alien aquatic plants and focus on the identification of research gaps.

Methods

Study area

The study covers two important Central European bioregions, the Alpine (Carpathians) and the Pannonian bioregions (Figure 1). The Pannonian bioregion is situated in the southern lowlands of Slovakia and is characterised by a relatively warm and dry climate with mean annual temperatures > 9 °C and relatively low total precipitation (< 600 mm). Conversely, a colder and more humid climate is typical for the Carpathians (mean annual temperatures 0–9 °C and total precipitation 600–1600 (2000) mm), which cover mainly large mountain ranges and inner-Carpathian basins in the central and northern parts. The area is very geologically heterogeneous and characterised by brackish and freshwater basin deposits in the south, flysch facies in the north and Mesozoic, marine and continental Triassic bedrocks in the central part (Miklós 2002).

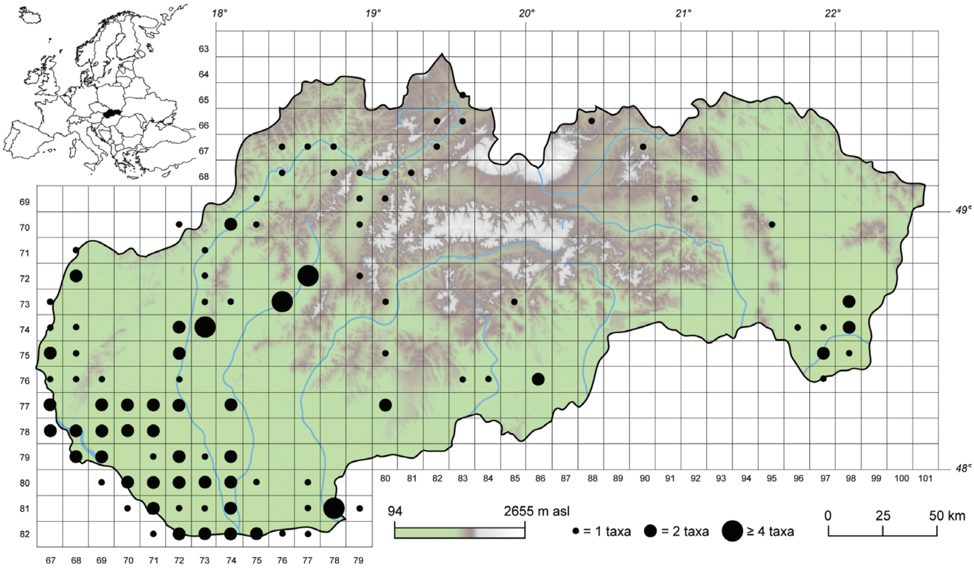

Figure 1. Spatial distribution of alien aquatic plants in Slovakia at the scale of the Central European Flora Mapping System.

The majority of water bodies in Slovakia belong to the catchment basin of the Danube River (Black Sea drainage area), while a small part (the Poprad River) flows to the catchment basin of the Vistula River (Baltic Sea drainage area). The majority of lotic water bodies in Slovakia have been heavily modified in the last century (Čiliak et al. 2014) and artificial canals and man-made lentic water bodies (e.g. gravel or sand ponds, water reservoir used for irrigation or recreation) have been constructed frequently. Therefore, together with thermal waters (small ponds or canals), artificial or human-modified water bodies create numerous habitats, potentially suitable for alien aquatic plants.

Data sources

We focused on alien aquatic vascular plant species using the definitions of alien species by Pyšek et al. (2004) and Blackburn et al. (2011). Aquatic plants were identified as those species that grow submerged or floating on the water surface for at least a part of their life history (Hussner 2012) and these included true aquatic plants (hydrophytes) and amphibious plants, adapted to both aquatic and terrestrial modes of life (cf. Janauer 2003, Janauer and Dokulil 2006). However, typical helophytes were excluded from the dataset.

We established a database of alien aquatic plants, based on data from the Database of non-native plant species of Slovakia (http://dass.sav.sk/en/) and a checklist of alien flora of Slovakia (Medvecká et al. 2012). After critical review, we added data from the Central database of phytocenological relevés of Slovakia (http://ibot.sav.sk/cdf/) up to 2016, scientific articles (Suppl. material 1), nature-based web sites (https://fotonet.sk/), (https://www.nahuby.sk/) and herbaria (BP, BRA, SAV, SLO, SMBB, OLM, PMK, WU; for acronyms, see http://sweetgum.nybg.org/science/ih/ and Vozárová and Sútorý 2001). Last but not least, a large unpublished dataset, gathered by the authors during the intensive research of all types of water bodies from 2011–2017, was included in the database. We performed extensive floristic, phytosociological and ecological surveys of aquatic habitats in understudied parts of Slovakia (cf. Baláži et al. 2011). Besides native species, a large amount of data on alien aquatic plants was gathered during the research (e.g. Kochjarová et al. 2013, Bubíková et al. 2016, Nobis et al. 2019). Altogether, the database of alien aquatic plants in Slovakia contained 599 records. Collected data were further processed and multiple records for the same species in the same site over several years were reduced to a single oldest record. The database covered 512 unique records from 479 sampling sites. The records were arranged into grid cells according to the Central European Flora Mapping System (CEFMS, Niklfeld 1971). Whenever available, the plant data were supplemented by information on climate, landscape composition, habitat type and local physical and chemical conditions of water bodies.

Climate data (mean annual air temperature, January and July mean air temperatures and total annual precipitation) were calculated as mean values for the period 1981–2010. The data were extracted from raster layers provided by the Slovak Hydrometeorological Institute using the GRASS geographic information system (Grass Development Team 2010).

Composition of landscape was derived from CORINE Land Cover maps (Büttner and Kosztra 2017). We specifically focused on the coverage of road networks (thereafter also road networks), coverage of urban areas (urban areas) and on the proportion of forests, natural and semi-natural areas (natural areas) representing proxies for human-mediated vectors of dispersal, permanent human presence and intensity of land use, respectively, which are known to drive distribution of alien aquatic plants (e.g. Kelly et al. 2014; Tamayo and Olden 2014; Rodríguez-Merino et al. 2018). Land cover of those categories was calculated for each grid cell of the CEFMS in QGIS v. 3.6 (QGIS Development Team 2019).

Water bodies were classified according to their habitat type (lentic, lotic) and origin (natural: rivers, streams, river oxbows, watered terrain depression; artificial: drainage and irrigation canals, water reservoirs, sand or gravel pits). Local characteristics of water bodies, known to affect aquatic plant communities (Lacoul and Freedman 2006), were measured in the field as follows: the mean depth of water was calculated from 10 random measurements at each site; and water temperature, pH and conductivity were measured using a EUTECH Cyber Scan series 600 instrument. These local parameters were available only for 117 sites (103 sites with water depth, 68 with temperature, 75 with pH and 74 sites with conductivity values).

For each plant species, the first time of observation (FTO) and the following categories were evaluated: invasion status (IS), cas – casual, nat – naturalised, inv – invasive (Richardson et al. 2000); residence time (RT), arch – archaeophyte, neo – neophyte (Richardson et al. 2000); introduction mode (IM), d – deliberate, a – accidental, b – both means (Hulme et al. 2008, simplified according to Medvecká et al. 2012) and water type (WT), cold and thermal.

Data analysis

We constructed an analytical sample-based rarefaction curve with unconditional confidence intervals (Colwell et al. 2004) to assess the completeness of the inventory of alien aquatic plant species in Slovakia. The bias-corrected asymptotic species richness estimator Chao2-bc (Chao 2005) was used to estimate the total number of alien species, including those unobserved.

We evaluated the effects of climatic characteristics (mean annual air temperature, January and July mean air temperatures and total annual precipitation) and landscape characteristics (cover of road networks, urban areas and natural areas) on the diversity of alien aquatic plants using generalised linear models (GLMs, McCullagh and Nelder 1989). Prior to the analysis, we imposed a grid of the CEFMS over the studied area and pooled site-specific records for each grid cell. The grid cells were treated as sampling units in the GLMs to overcome possible problems with non-independence (e.g. sampling of several sites over a relatively short stretch of the same stream) and uncertainty in exact georeferencing of some historical records. Floristic records from thermal waters were excluded from this analysis since the occurrence of those species of (sub)tropical origin with higher temperature optima is mainly driven by locally-specific temperature regimes of water bodies without a direct link to regional climate or landscape features (cf. Vojtkó et al. 2017). Due to strong correlations amongst variables, we fitted separate GLMs for each predictor. The number of sampling sites in each grid cell was included as a covariate in the GLMs to account for differences in sampling effort amongst grids. Since the alien species counts showed lower variation than expected under the mean-variance relationship of the Poisson distribution (dispersion parameters of the Poisson GLMs $\varphi \sim 0.2$), we fitted GLMs with a Conway-Maxwell-Poisson distribution, a two-parameter generalised form of the Poisson distribution that is sufficiently flexible to describe count data with a wide range of dispersion levels (Shmueli et al. 2005). Diagnostic plots of residuals were inspected to assess the quality of the models and no violation of the assumptions was observed. The residuals were also screened for spatial autocorrelation using non-parametric spatial correlograms (Bjørnstad and Falck 2001) and any significant autocorrelation patterns were detected. Finally, a leave-one-out cross-validation procedure was employed to assess the predictive performance of the GLMs based on median absolute errors (MdAE).

Since the majority of records in the database stem from unstructured, opportunistic (presence-only) sampling lacking site-specific environmental information and since many species were found in only a few sites, we did not use inferential statistics to estimate species habitat preferences. Instead, we relied on exploratory data analysis and used a series of bar plots and boxplots to examine the environmental tolerances of alien aquatic plants in Slovakia. In particular, we focused on optima (median) and ranges (min-max) of species with a sufficient number of records.

The analyses were performed in Spade (Chao and Shen 2010) and R (R Development Core Team 2018) using the packages COMPoissonReg (Sellers et al. 2017), ggplot2 (Wickham 2016), iNEXT (Chao et al. 2014) and ncf (Bjørnstad 2018).

Results

Inventory of alien aquatic plant species in Slovakia

Altogether, twenty alien aquatic plant taxa were recorded in Slovakia (Table 1). The historical overview showed a strong increasing trend in the number of alien species over the last 30 years (Figure 2A). Indeed, the trend is parallel to the degree of scientific interest in alien plants mirrored in a number of published studies. However, the species inventory seems to be far from complete, as is apparent from the non-asymptotic rarefaction curve (Figure 2B). The expected total number of alien aquatic species calculated by the Chao2-bc estimator is 34 (95% conf. interval: 23–87), which means that 14 species are expected to remain undetected.

All of the recorded aliens belong to neophytes and a substantial proportion has naturalised invasion status (70%) and a deliberate introduction mode (60%) (Table 1). *Elodea canadensis* and *E. nuttallii* were the most widespread (55 and 41 grid cells, respectively) followed by a *Nymphaea* cultivar, *Pistia stratiotes*, *Azolla filiculoides*, *Eichhornia crassipes* and *Najas guadalupensis* (> 6 grid cells), while the remaining 65% of species occurred infrequently (≤ 3 cells).

The effect of climate and landscape characteristics on the diversity of aliens

Alien aquatic plants were recorded in 98 grid cells of the CEFMS (~23% of all cells), mainly in the lowlands and valleys of large rivers (Figure 1). This geographic pattern corresponds well with the results of GLMs. All studied climatic variables were significantly related to the number of alien species after accounting for sampling effort (Table 2). The diversity of alien species increased with temperature and decreased with precipitation. In addition, the number of aliens significantly increased with decreasing cover of natural and semi-natural areas. When we combined best climatic (mean annual temperature) and landscape (natural areas) predictors in a single model, predictive performance improved over those simple GLMs (MdAE = 0.30). However, there is still a lot of unexplained variance in the data (Figure 3).

Habitat preferences of aliens

A comparable number of species was found in cold and thermal waters (Table 1). All but one alien species (*Lemna turionifera*) were found in artificial water bodies and 60% of them were recorded exclusively in man-made habitats (Figure 4). Half of the species were able to colonise both lotic and lentic habitats, while 35% and 15% were found only in standing or running waters, respectively. Considering temperature preferences, *Hydrilla verticillata*, *Najas guadalupensis* and *Sagittaria subulata* prefer warm waters, while the other evaluated taxa (*Elodea canadensis*, *E. nuttallii*, *Nymphaea* sp. and *Pistia stratiotes*) were found in a relatively wide range of temperatures. Regarding water conductivity, the examined plants occurred in waters with an average to high mineral content (90–2790 µS/cm). *Elodea canadensis*, *Nymphaea* cultivar and *Najas guadalupensis* were the only taxa that were occasionally found in slightly acidic waters. Habitats of the other species showed neutral to alkaline pH. Amongst the five species with available water depth data, only *Elodea* species were also able to dwell in deeper waters (> 2 m). The remaining alien plants were recorded in shallow or even very shallow waters (< 0.4 m, *Sagittaria subulata*).

Table 1. List of the alien aquatic plants in Slovakia.

Species / family	FTO	Source of FTO	IS	RT	IM	WT	GO	CEFMS
Alisma subcordatum Raf. / Alismataceae	2017	Hrivnák observed & photo	cas	neo	d	Cold	Am	1
Azolla filiculoides Lam. / Salviniaceae	1951	Hejný (1958)	nat	neo	a	Cold	Am	9
Egeria densa Planch. / Hydrocharitaceae	1993	Somogyi (1995)	nat	neo	d	Therm	Am	1
Eichhornia crassipes (Mart.) Solms / Pontederiaceae	1999	Ružičková (2000)	nat	neo	d	Cold	Am	7
Elodea canadensis Michx. / Hydrocharitaceae	1883	Arpád Degen, BP	nat	neo	a	Cold	Am	58
Elodea nuttallii (Planch.) H. St. John / Hydrocharitaceae	1986	Helena Oťaheľová, SAV	nat	neo	a	Cold	Am	42
Hydrilla verticillata (L. f.) Royle / Hydrocharitaceae	1995	Májský and Rusko (1999)	nat	neo	d	Therm	As	2
Lemna minuta Kunth / Lemnaceae	1997	Feráková and Onderíková (1998)	nat	neo	a	Cold	Am	1
Lemna turionifera Landolt / Lemnaceae	2006	Helena Oťaheľová, CDPR	cas	neo	a	Cold	Am, As	1
Limnophila sessiliflora Blume / Plantaginaceae	1993	Somogyi (1995)	cas	neo	d	Therm	As	1
Ludwigia repens J. R. Forst. / Onagraceae	2017	Nobis et al. (2019)	nat	neo	d	Therm	Am	1
Najas guadalupensis (Spreng.) Magnus / Hydrocharitaceae	1986	Feráková and Kocianová (1997)	nat	neo	d	Both	Am, As	6
Nymphaea L. (cultivar) / Nymphaeaceae	1998	Májský and Rusko (1999)	nat	neo	d	Both	Unk	17
Pistia stratiotes L. / Araceae	2007	Tóthová et al. (2011)	nat	neo	b	Both	Am	10
Sagittaria latifolia Willd. / Alismataceae Vent.	2013	Nobis et al. (2019)	cas	neo	b	Cold	Am	3
Sagittaria subulata (L.) Buchenau / Alismataceae	1995	Májský and Rusko (1999)	nat	neo	d	Therm	Am	3
Shinnersia rivularis (A. Gray) R. M. King & H. Rob. / Asteraceae	1998	Májský and Rusko (1999)	nat	neo	d	Therm	Am	1
Utricularia gibba L. / Lentibulariaceae	1993	Somogyi (1995)	nat	neo	d	Therm	Am, As	1
Vallisneria spiralis L. / Hydrocharitaceae	2011	Košťál in Eliáš (2012)	cas	neo	d	Cold	Af, Am, As	1
Victoria amazonica Sowerby / Nymphaeaceae	1998	Májský and Rusko (1999)	nat	neo	d	Therm	Am	2

Legend: FTO – first time of observation; IS – invasion status, cas – casual, nat – naturalised, inv – invasive; RT – residence time; neo – neophyte; IM – introduction mode, d – deliberate, a – accidental, b – both means; WT – water types, Cold – freshwater, Therm – thermal water, Both – freshwater and thermal water; GO – geographical origin, Af – Africa, Am – America, As – Asia, Unk – unknown; BP – herbarium of the Hungarian Natural History Museum, CDPR – central database of phytocenological relevés of Slovakia, SAV – herbarium of the Institute of Botany, Slovak Academy of Sciences; CEFMS – number of Central European Flora Mapping System grid cells occupied by a species.

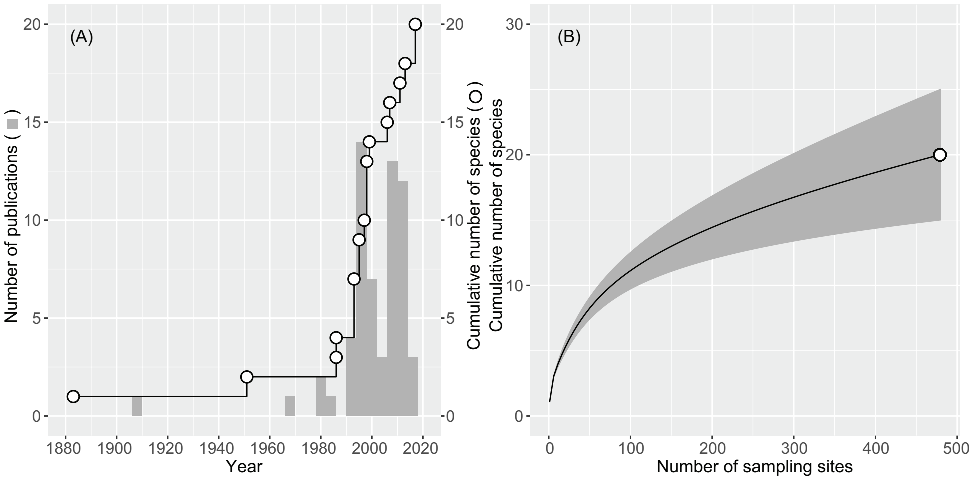

Figure 2. A Temporal trend in the number of studies involving alien aquatic plants (grey histogram) and cumulative number of alien aquatic plants recorded in Slovakia **B** Sample-based rarefaction curve of the number of alien aquatic plant species in Slovakia. The grey area represents the 95% confidence band of the diversity estimate. Full list of studies is given in Suppl. material 1.

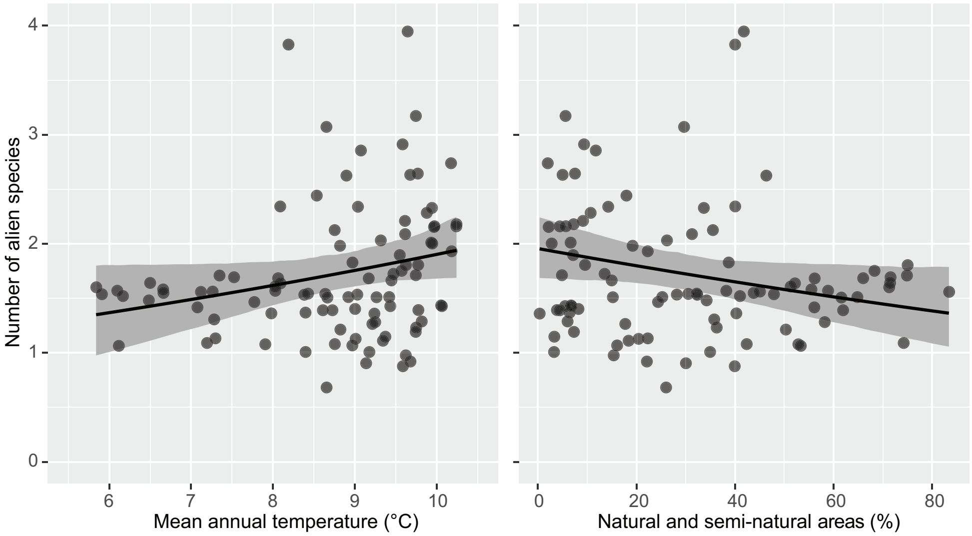

Figure 3. Conwell-Maxwell-Poisson GLM showing a partial relationship between mean annual air temperature, coverage of natural and semi-natural areas and the number of alien aquatic plant species recorded at the scale of the Central European Flora Mapping System with the sampling effort constant at a mean of 4.9 sites. The predicted number of species (line), 95% bootstrap confidence intervals (grey polygon) and partial residuals (points) are displayed.

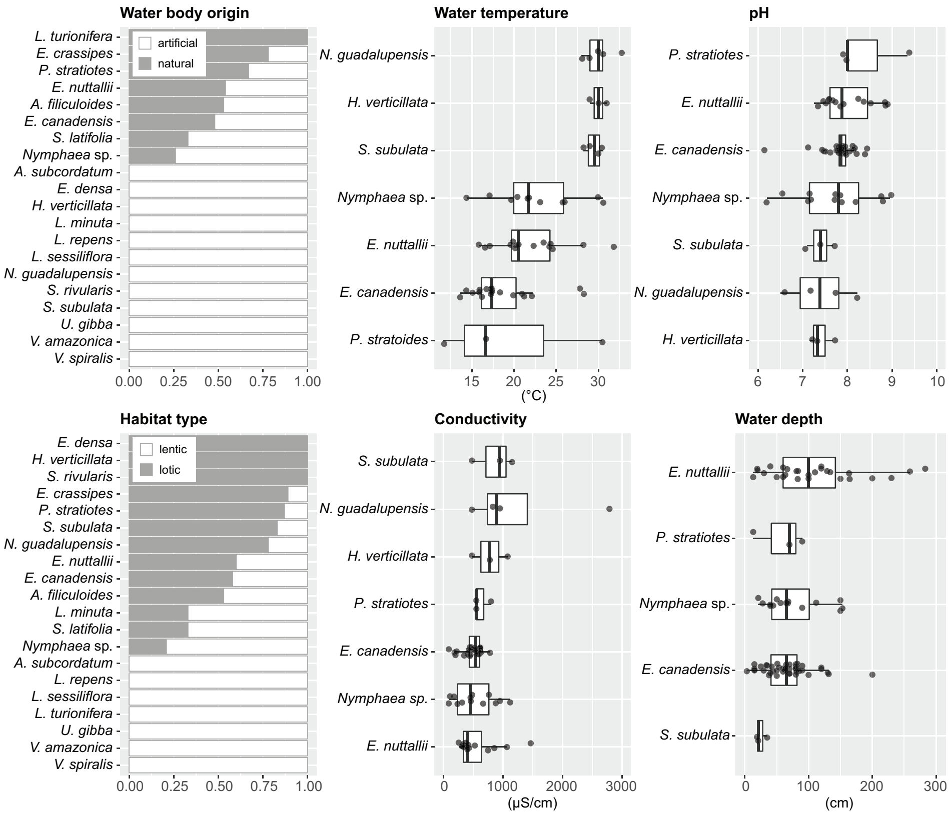

Figure 4. Environmental preferences of alien aquatic plants observed in Slovakia. Bar plots display the relative occupancy of water bodies according to the origin (artificial, natural) and habitat type (lotic, lentic). Boxplots show the occurrence of alien aquatic plants along environmental gradients of water temperature, conductivity, pH and water depth. Only species with at least 3 environmental measurements are plotted. Boxplots display median (line), interquartile range (box), range (whiskers) and observed values (jittered points). Full names of taxa are presented in Table 1.

Table 2. Results of Conway-Maxwell-Poisson GLMs for the effect of climatic and landscape characteristics on the number of alien aquatic plants in the grid cells of the Central European Flora Mapping System. Standardised regression coefficients (β) and dispersion parameters (ν) are displayed along with their 95% bootstrap confidence intervals (95%CI), test statistics (z, χ^2) and probabilities (p). The cross-validated median absolute error of prediction (MdAE) is shown for each model.

Environmental variables	Model coefficients			Dispersion parameters			MdAE
	β (95%CI)	z	p	ν (95%CI)	$\chi^2_{(1)}$	p	
Climate							
Mean annual temperature	0.83 (0.39–1.49)	3.22	0.0013	6.47 (5.12–9.17)	83.79	< 0.0001	0.332
Mean July temperature	0.81 (0.36–1.42)	3.19	0.0014	6.44 (5.02–9.03)	83.56	< 0.0001	0.333
Mean January temperature	0.81 (0.37–1.41)	3.17	0.0015	6.41 (5.10–9.08)	83.26	< 0.0001	0.337
Annual precipitation	-0.88 (-1.59– -0.45)	-3.32	0.0009	6.63 (5.29–9.49)	84.97	< 0.0001	0.381
Landscape							
Road networks	-0.25 (-0.57–0.19)	-0.62	0.5360	5.57 (4.45–7.92)	73.86	< 0.0001	0.421
Urban areas	0.44 (-0.10–0.70)	1.41	0.1579	5.68 (4.43–7.96)	75.15	< 0.0001	0.371
Natural areas	-0.79 (-1.34– -0.39)	-3.32	0.0009	6.48 (4.79–9.43)	84.51	< 0.0001	0.371

Discussion

Inventory of alien aquatic plant species in Slovakia

Our review of published and unpublished data revealed the presence of 20 alien aquatic plant species in Slovakia. The number of recorded species has steeply increased with scientific interest in recent decades (Figure 2). The spatial distribution of alien aquatic plants in Europe shows an uneven pattern (Hussner 2012), which does not fully correspond to the general picture of the climate-driven distribution of alien plants in Europe (Chytrý et al. 2009). Specifically, the highest number of species is known from Italy, France, Germany, Belgium, Hungary, Greece and the Netherlands (Hussner 2012; Brundu et al. 2013; Lansdown et al. 2016). This irregular pattern apparently relates to the intensity of aquatic vegetation research. For example, some southern European countries with Mediterranean climates, such as Albania, Bosnia or Montenegro, lack alien aquatic plant studies, which results in a seemingly low diversity of aliens in the waters (Hussner 2012; Lansdown et al. 2016). In contrast, comprehensive research can reveal surprising results. For example, Hungary hosts 48 species, which represents almost half of the known alien aquatic plants in Europe (Lukács et al. 2016). The high importance of sampling effort is obvious in the case of Slovakia. Intensive research in recent years has led to a steep increase in the number of alien aquatic plants on the national checklist and the last published inventories (Hussner 2012; Medvecká et al. 2012) are therefore outdated. In a broad context, high regional differences in the state of knowledge and research intensity may obscure or even preclude large-scale syntheses on the distribution of alien aquatic plants in Europe.

Given the occurrence of many rare species (singletons and doubletons) in Slovakia, the total number of alien aquatic plants is expected to be much higher (Chao2-bc = 34 species) than observed. We may reasonably assume the presence of several aliens, such as *Cabomba caroliniana* A. Gray, *Elodea callitrichoides* (Rich.) Casp., *Hydrocotyle ranunculoides* L. f., *Lagarosiphon major* (Ridl.) moss or *Pontederia cordata* L., reported from neighbouring countries. For example, *C. caroliniana* has been established for a long time in the Pannonian lowlands (Lukács et al. 2016) occurring along the main river course of the Danube River and along several canals in central Hungary (Király et al. 2008) as well as in a few isolated sites, including the Danube River at the Slovak-Hungary border (Bartha and Király 2015). Similarly, *L. major* and *P. cordata* are known from Hungary and the Czech Republic in the regions bordering Slovakia (Bartha and Király 2015; Kaplan et al. 2016). Other species that are frequent in Europe (e.g. *Crassula helmsii*, *Myriophyllum aquaticum* (Vell.) Verdc.) might also be overlooked or their presence may be limited by specific habitat requirements, which are rarely found in Slovakia (Dawson and Warman 1987; (https://www.cabi.org/ISC/datasheet/16463); (https://www.cabi.org/isc/datasheet/34939); Kasper and Krausch 2008).

Moreover, a broad number of alien aquatic species, mainly aquarium and ornamental plants, could be added to the list of alien aquatic plants in the future due to their potential release to thermal waters, such as small ponds and fountains in thermal spas, canals with thermal wastewater from spas and swimming pools and/or aquarium waste. The list of these species depends on trade by aquarium and gardening shops. Generally, the pet/aquarium/terrarium trade is responsible for the introduction of numerous alien plants (Padilla and Williams 2004; Brunel 2009). This introduction mode is responsible for the spread of a substantial portion of alien aquatic plants in Europe and America (Maki and Galatovitsch 2004; Hussner et al. 2010; Peres et al. 2018) and was also a main mode of introduction in our study. Therefore, raising awareness about the harmful effects of dumping alien plant species to natural habitats is an important message to the public with the aim of preventing these activities.

Finally, it should be noted that some alien aquatic plants found in Slovakia are considered as invasive alien species of European Union concern (e.g. *Eichhornia crassipes*, *Elodea nuttallii*) and they require legislative attention and adequate prevention and management of their introduction and spread on a national level, as stated in EU Regulation no. 1143/2014.

The effect of climate and landscape characteristics on the diversity of aliens

We have shown that the diversity of alien aquatic plants is significantly linked with climatic conditions. In particular, the number of species increases along gradients of increasing air temperatures and decreasing precipitations. The geographic ranges of many alien aquatic plant species are strongly associated with climatic tolerances set by air temperatures (Kelly et al. 2014; Rodríguez-Merino et al. 2018) and a large number of studies have predicted alien species range shifts and expansions related to climate change (Bellard et al. 2018). In addition, the establishment of viable populations may be limited by temperature-controlled seed production and germination (Vojtkó et al. 2017).

The role of precipitation is less obvious since temperature characteristics and precipitation were strongly correlated in the studied area (Pearson r = -0.78 – -0.87). However, if we combined temperatures and precipitation in a single model or if we used some compound measures, such as climatic moisture index (Willmott and Feddema 1992), predictive performance would be comparable or even worse than in the case of simple temperature models. In other words, beside temperatures, precipitation did not contribute any additional information useful for predictions of alien species diversity. Since a vast majority of the investigated water bodies are permanent with a relatively stable water level, we believe that precipitation does not constrain distribution of alien aquatic plants in the region, as suggested from the grid-level data.

Our results also revealed that landscape with a higher proportion of natural and semi-natural areas supports lower diversity of aliens than intensively managed land. However, we have also shown that plain habitat accessibility to humans, as vectors of dispersal, is not sufficient to explain diversity patterns of aliens, since neither road network coverage nor the proportion of urban areas alone were significantly related with the alien species diversity. Human-mediated landscape effects are likely more complex, involving both accessibility and intensive land use. For example, extensive agricultural cultivation, associated with irrigation channels and elevated nutrient levels, may facilitate dispersal and establishment of alien aquatic plant populations (Téllez et al. 2008; Rodríguez-Merino et al. 2018). Similarly, Kelly et al. (2014) identified land use, nutrient levels and natural landscape as the most important factors associated with alien aquatic species ranges at the regional level. Tamayo and Olden (2014) also found that the probability of lake invasion by noxious submerged macrophytes is positively linked with the intensity of land use in the surrounding habitats. Apparently, the areas at greatest risk of invasions by aquatic plants in Europe are those experiencing considerable human pressure (Rodríguez-Merino et al. 2018).

In conclusion, our results reaffirmed the major role of climate and landscape modification in the distribution of alien aquatic plants. We may reasonably expect further increases in alien numbers under ongoing global climate change and land use intensification, especially in the lowlands of southern and eastern Slovakia. Moreover, since elevated temperatures and CO_2 levels are assumed to increase the performance of alien plants more steeply than that of native species (Sorte et al. 2013), aquatic systems may be particularly vulnerable to invasion as climate change proceeds and alien plant species may exert a stronger pressure on native biodiversity and ecosystem functioning than previously thought.

Habitat preferences of aliens

The lack of detailed information on local environmental conditions hampered our ability to draw broad conclusions about the habitat preferences of alien aquatic plant species in Slovakia. However, a few consistent patterns emerged. First, artificial water bodies were more often colonised by alien species than natural habitats and the majority of the species were found exclusively in man-made water bodies. Indeed, this seemingly higher preference of alien species for artificial habitats may partly stem from the fact that many (sub)tropical species are inevitably present only in artificial water bodies with thermal water (e.g. wastewater canals from thermal spas). However, our observations are in agreement with the patterns recorded in the terrestrial realm, where heavily modified and man-made habitats rank amongst the most invaded biotopes in Europe (Chytrý et al. 2009; Medvecká et al. 2014). Disturbed systems are generally more susceptible to invasions due to elevated fluctuations in resource availability (Davis et al. 2000; Hussner et al. 2017). Lower competition by native species in artificial habitats might also play a role (biotic resistance hypothesis, Levine et al. 2004), although evidence for this mechanism is rather weak in freshwaters (Alofs and Jackson 2014; Svitok et al. 2018).

Second, species with available environmental information showed relatively wide environmental tolerances (Figure 4), i.e. they were able to colonise both standing and running waters, tolerate a wide range of pH and conductivity values and, except for (sub)tropical species, span a large gradient of water temperatures. In general, environmental tolerance is a key parameter in the establishment success of introduced alien species (van Kleunen et al. 2015). Svitok et al. (2018) stated that alien aquatic plants have broad niches, while invaded aquatic environments may not possess environmental constraints that are strong enough to filter alien macrophytes. Consequently, the presence and diversity of aliens may be difficult to predict using habitat properties.

Finally, our research revealed a serious gap in knowledge of alien aquatic plant habitat requirements; only a few species have sufficient records of local habitat quality necessary for sound examination of environmental niches. Therefore, further research should focus on estimating environmental niche breadths and subsequently identifying the potential invasiveness of alien aquatic plants.

Conclusions

Based on a thorough review of published and unpublished resources, 20 alien aquatic species were recorded in Slovakia. However, the presence of many other alien species might be reasonably expected considering (i) a high proportion of rare species (low detectability), (ii) the deliberate introduction of aquarium and ornamental plants and (iii) the positive effect of rising temperatures and intensively modified landscape on alien species diversity. Given ongoing climate change and land use intensification, one can reasonably assume enhanced invasiveness and spreading of alien species into new habitats.

Filling a gap in the recognition of alien aquatic plant environmental tolerances is a challenge for future research. There is also an urgent need for studies on population dynamics, reproductive output, seed-bank characteristics and functional traits of alien aquatic vascular plants, as well as their competitive ability and their interactions with native biota in freshwaters. Finally, raising public awareness and developing adequate management strategies are ultimate conservation goals for maintaining natural aquatic plant diversity and ecosystem functioning.

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Supplementary material 1

List of references used for the preparation of a database of alien aquatic plants in Slovakia

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RESEARCH ARTICLE

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Impacts of non-native fishes under a seasonal temperature gradient are forecasted using functional responses and abundances

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Abstract

Developing predictive methods to forecast the impacts of existing and emerging invasive species is of critical importance to biodiversity conservation. However, invader impacts are context-dependent, making reliable and robust predictions challenging. In particular, it is unclear how temporal variabilities in relation to temperature regime shifts influence invader ecological impacts. In the present study, we quantify the functional responses of three coexisting freshwater fishes: the native freshwater River Goby *Glossogobius callidus*, and the non-native Mozambique Tilapia *Oreochromis mossambicus* and Western Mosquitofish *Gambusia affinis*, under two temperature treatments using chironomid larvae as prey. This was used along with fish abundance data to determine temporal differences in ecological impacts of each fish species between seasons (i.e. at two corresponding temperatures). All three fish species exhibited potentially population-destabilizing Type II functional responses. Their maximum feeding rates were consistently higher in the warm temperature treatment, whereas attack rates tended to be reduced. Non-native Mozambique Tilapia had the highest maximum feeding rate under both temperature treatments

(18 °C and 25 °C), followed by the non-native Western Mosquitofish and lastly the native River Goby, suggesting greater *per capita* impacts on native prey by non-native fishes. The predatory fish abundances differed significantly according to season, with native River Goby and non-native Mozambique Tilapia generally more abundant than non-native Western Mosquitofish. By multiplying functional response maximum feeding rates with abundances of each fish species across the seasonal gradient, the relative impact potential of non-native Mozambique Tilapia was consistently higher compared to that of native gobies. Western Mosquitofish impacts were less apparent, owing to their low abundances. We demonstrate how seasonal temperature fluctuations affect the relative impact capacities of introduced species and the utility of consumer functional response and the relative impact potential metric in impact forecasting.

Keywords
Context-dependence, impact assessment, introduced species, relative impact potential, seasonal abundance, thermal regime

Introduction

Biological invasions are a central driver of global biodiversity loss (Sala et al. 2000; Turak et al. 2016; Bertelsmeier and Keller 2018; Shuai et al. 2018). This loss has not only socio-economic impacts, but also threatens ecosystem functions and services (Ricciardi et al. 2017). Biological invasions can occur through numerous pathways, such as human-mediated introduction, climate change and connectivity of systems, thus allowing extra-limital movement of species (Latombe et al. 2017). Upon arrival in a new environment, non-native species can cause ecological impact on native species assemblages through a range of biotic interactions (e.g. predation, competition and parasitism) (Vitousek et al. 1996; Thomsen et al. 2011; Havel et al. 2015; Seebens et al. 2018). Competition and predation play particularly important roles in the structuring of ecological communities (Paine 1980; Gurevitch et al. 1992). Although the impact of invaders through predation and competition is well documented, the context-dependency of these processes is often overlooked. In particular, direct biotic interactions (i.e. predation) can drive trophic cascades through alterations of prey abundance and native predator fitness (Gallardo et al. 2016; Penk et al. 2017).

Despite the considerable work conducted on invasive species, predicting ecological impacts of biological invasions has remained elusive (Simberloff et al. 2013; Dick et al. 2014). Ricciardi et al. (2013) highlighted context-dependency as the largest confound for impact predictions in invasion biology. Therefore, robust predictive methods that include environmental contexts as factors are needed in invasion studies to improve impact forecasting. In particular, temperature regime is a key abiotic context that is pervasive across all ecosystem types, and particularly in aquatic ecosystems (Lang et al. 2017). Specifically, temperature is a central determinant of the strength of predator-prey interactions and mediates food web stability (Rall et al. 2010, 2012; Englund et al. 2011). Fish have physiological mechanisms (i.e. metabolism and reproductive success) that are directly and/or indirectly dependent on temperature (Roessig et al. 2004). These mechanisms may differ between native and non-native species given differences in geographical origins and their physiological tolerances (Sorte et al. 2013). If high temperatures are more physiologically optimal for invaders, ecological impacts may be intensified (Iacarella et al. 2015), and seasonal changes, coupled with ongoing climatic warming, are key drivers of such temperature change in aquatic ecosystems. Indeed, interaction strengths are known to vary even with slight changes to seasonal temperatures (Sanford 1999, 2002). Therefore, failure to incorporate these factors in predictive approaches limits our ability to forecast invasive species impacts under changing environmental conditions across different spatiotemporal scales (Dick et al. 2013, 2014, 2017).

Methodological developments, which incorporate native/non-native species resource utilization across context-dependencies, have recently provided robust predictive tools for invasion science (Laverty et al. 2015; Dick et al. 2017; Dickey et al. 2018; Cuthbert et al. 2019). In particular, the functional response quantifies resource consumption as a function of resource density, and, in a predator-prey context, can quantify *per capita* ecological impacts of predators towards lower trophic groups (Holling 1959; Adams 1980; Dick et al. 2013, 2014, 2017; Alexander et al. 2014; Cuthbert et al. 2018). The types and magnitude of FRs quantify whether consumers will likely stabilize or destabilize resource populations (Murdoch and Oaten 1975; Rip and McCann 2011; Uszko et al. 2017). The relationships between consumer resource uptake and resource densities results in three broad functional response 'Types', and each Type has a different effect to resource population stability: an increasing linear relationship with no handling time constraint (Type I, mechanistically exclusive to filter feeders; Jeschke et al. 2004); an inversely density-dependent response characterized by high resource consumption at low resource density (Type II, resulting in rapid resource depletion at low densities); and a sigmoidal positively density dependent relationship (Type III, where resources have a low-density refuge) (Holling 1959). Despite the two functional response components, i.e. attack rate and handling time, being strongly associated with variations in temperature (Englund et al. 2011; Rall et al. 2012; Sentis et al. 2012; South et al. 2017; Cuthbert et al. 2018), there is, however, very limited information available on how temperature mediates species interactions at the population-level (Viherluoto and Viitasalo 2001; Fussmann et al. 2014; O'Gorman et al. 2017). Temperature and/or season effects may differ depending on how species functional traits directly influence responses, and these traits may too change along environmental gradients (Chapin et al. 2000).

Classically, the functional response has been combined with the 'numerical response' to determine the 'total response' of consumers (Solomon 1949; Holling 1959). The numerical response describes the consumer population-level response to changes in resource densities, while 'total response' can be defined as the multiplication of species' numerical response with functional response (Solomon 1949; Holling 1959). Given that the numerical response, in comparison to the functional response, is difficult to ascertain, consumer abundance has recently been proposed as a proxy for numerical response in the development of the 'impact potential' and 'relative impact potential' metrics (Dick et al. 2017; Dickey et al. 2018). The 'impact potential' is the product of functional responses and abundance of consumers, while the relative impact potential compares the impact of the invader to that of a native (Dick et al. 2017). The strength of the relative impact potential metric lies in its ability to incorporate both species abundance and functional response under different environmental conditions (e.g. temperature change) and thus predict the influence of context-dependencies on invader impact (Laverty et al. 2017). This metric provides a novel approach for assessing existing and potential ecologically damaging species through the use of actual field abundance data under different environmental conditions.

The current study focuses on one native and two non-native fish species that co-occur in irrigation ponds within the Sundays River Valley, Eastern Cape, South Africa. These are the native River Goby *Glossogobius callidus* (Smith, 1937), and two non-native species, Mozambique Tilapia *Oreochromis mossambicus* (Peters, 1852) and Western Mosquitofish *Gambusia affinis* (Baird and Girard, 1853). The native River Goby is naturally found in estuarine and freshwater habitats (Engelbrecht and Mulder 1999; James et al. 2007). The River Goby is an invertivorous species (Wasserman 2012; Mofu et al. 2019). The non-native Mozambique Tilapia is native to eastward flowing rivers of central and southern Africa but its natural distribution does not extend to the Sundays River (Skelton 2001). The non-native Mozambique Tilapia is an omnivorous species, with clear ontogenetic shifts in diet, where juveniles feed predominantly on zooplankton and insects, while the diet of adults comprises of vegetative detritus (Zengeya et al. 2011). The non-native Western Mosquitofish is native to the lowland ponds, lakes and drainages of North America from Mexico to Alabama (Skelton 2001; Pyke 2008). It is an opportunistic omnivore feeding on algae, crustaceans, insects and amphibian larvae (Pyke 2008). Both non-native Mozambique Tilapia and Western Mosquitofish have been listed in the top 100 worst global invasive species database (IUCN 2006). Given that these three fish co-occur and are the most abundant within the Sundays River Valley irrigation ponds, this study sought to comparatively assess the potential relative ecological impacts of non-native Mozambique Tilapia and non-native Western Mosquitofish relative to the native River Goby towards native benthic prey across a seasonal temperature gradient.

Materials and methods

Ethical clearance and permits

The collection of animals and all experiments were carried out in compliance with the Eastern Cape Department of Economic Development and Environmental Affairs (DEDEA permit no. CRO 35/17CR and CRO 36/17CR) and ethical clearance was approved by the National Research Foundation – South African Institute for Aquatic Biodiversity (NRF-SAIAB reference no. 25/4/1/5_2017/03).

Functional response experimental design

River Goby, Mozambique Tilapia and Western Mosquitofish individuals were sourced using a 30 m × 2 m seine net with 12 mm mesh wings and an 8 mm mesh cod-end from

Dunbrody (33°27'53"S; 25°33'02"E) and Disco Chicks (33°27'26"S; 25°39'57"E) irrigation ponds, Eastern Cape, South Africa. Upon capture, fish were transported to NRF-SAIAB, Grahamstown in continuously aerated containers with source water. Each fish species was housed separately in a controlled temperature and light laboratory and kept under a 12:12 light:dark cycle. Temperature was maintained at either 18 ± 2 °C or 25 ± 2 °C (i.e. experimental temperature groups) for seven days prior to experimentation, with each species acclimated separately in 40 L fish tanks in a closed recirculating system. All fish were maintained on a standardized diet of larval chironomids *ad libitum*. The chironomid larvae were collected by kick sampling from the Bloukrans River (33°19'06"S; 26°34'22"E) using a kick net (1000 µm). The chironomids were then strained twice through 2.0 mm and then 1.0 mm sieves to obtain the experimental size class (total length (T_L) ± standard deviation ((SD) 1.5 ± 0.11 mm) and then rinsed thoroughly with deionized water to remove any other food sources.

Functional response experiments were performed at 18 °C and 25 °C, reflecting respective spring and summer temperatures at the sampling locations. Following Alexander et al. (2014), all fish were size matched (T_L (mean ± SD): River Goby = 41.50 ± 4.10 mm; Mozambique Tilapia = 41.70 ± 4.10 mm; Western Mosquitofish = 41.60 ± 4.10 mm), in order to eliminate the influence of size-related differences on prey consumption and focus on species-specific differences (Rall et al. 2012). Individuals of River Goby, Mozambique Tilapia and Western Mosquitofish were randomly selected from the holding tanks 24 hours prior to the trial and transferred to experimental arenas (opaque 20 L spherical arenas: diameter: 290 mm; depth: 400 mm) containing 5 L of continuously aerated rainwater. In individual experimental arenas, each assigned fish was held for 24 hrs prior to the experiment without food to allow for acclimatization and standardization of hunger levels. Individual fish were then presented with chironomid larvae at one of eight prey densities (n = 2, 4, 8, 16, 32, 64, 96 and 120; n = 7 replicates per prey density). At the end of each experimental period, predators were removed and the total number of live prey items remaining, and hence numbers consumed, enumerated. One set of experiments (i.e. one randomized fully factorial replicate per experimental temperature group) was conducted in a day, and the experiments were initiated at 09:00 am, during photoperiod, with prey consumption examined after 2 hrs. Controls consisted of larval chironomids in experimental tanks at each prey density in the absence of predators (n = 2 replicates per experimental group). Predators were only used once and therefore there was no re-use within or across experimental groups.

Fish abundances

The fish predator abundance data were obtained from the NRF-SAIAB`s monitoring program of irrigation ponds in the Sundays River Valley, Eastern Cape, South Africa. Abundance from two irrigation ponds were used, ML Swart (33°24'33"S; 25°29'04"E), and River Bend (33°26'23"S; 25°42'25"E). The pond names represent either the property or farm owner's name, as recorded by the Lower Sundays River Water User Association. These ponds were selected on the basis that they were surveyed in both spring and summer and that all three species were captured to give abundance estimates. During each survey, the irrigation pond water temperatures were measured using a HANNA HI98129 combo pH and electrical conductivity meter (HANNA Instruments Inc., Woonsocket, USA). Spring (18 °C) and summer (25 °C) abundance estimates were used in this study as they were in line with the experimental temperatures, and reflect seasonal temperature means.

The ponds were surveyed using a 30 m × 2 m siene net with 12 mm mesh wings and an 8 mm mesh cod-end. At least three hauls were conducted per pond and, upon completion of a single haul, all fish were kept alive in a continuously aerated container (20 L) until every seine haul was completed within a pond. Fish were then identified to species-level, enumerated and released back to the water. The abundance data were based on maximum catch field abundances using mean catch per 100 m^2.

Statistical analyses

Generalized linear models (GLMs) assuming a Poisson error distribution and log link were used to analyze overall prey consumption with respect to species, temperature and prey supply. Likewise, GLMs were used to compare fish abundances with respect to species, season and pond. Non-significant terms and interactions were removed stepwise to obtain minimum adequate models (Crawley 2007). Tukey's comparisons were used to undertake *post hoc* tests of significant effects in each resulting model (Hothorn et al. 2008).

To distinguish between Type II and III functional responses, logistic regression of the proportion of prey consumed as a function of initial prey density was performed (but see also Rosenbaum and Rall 2018). Selection between Type II and Type III models was further confirmed *via* comparison of Akaike's information criterion. A significantly negative first-order term indicates a Type II functional response, whereas a significantly positive first-order term followed by a significantly negative second-order term indicates a Type III response (Juliano 2001). Rogers' random predator equation was used to model functional responses as prey were not replaced as they were consumed (Rogers 1972):

$$N_e = N_0 \left(1 - \exp\left(a\left(N_e h - T\right)\right)\right) \tag{1}$$

where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack rate, h is the handling time and T is the experiment duration (fixed at 1). To enable model fitting, the *Lambert W* function was used (needed as N_e appears on both sides of the equation; (Bolker 2008)). Differences in attack and handling parameters were assessed pairwise between fishes at each temperature using indicator variables (Juliano 2001; Pritchard et al. 2017). Bonferroni corrections were used on raw p-values to account for multiple comparisons. Multiple estimates of the handling time parameter h were generated using non-parametric bootstrapping (n = 100), with maximum feeding rates then calculated *via* $1/h$.

We calculated relative impact potential (RIP) of native (i.e. River Goby) and non-native (i.e. Mozambique Tilapia, Western Mosquitofish) species using the mean bootstrapped functional response maximum feeding rate (FR) and abundance (AB) for the three species at each season and pond (Dick et al. 2017):

$$\mathrm{RIP} = \left(\frac{\text{FR non-native}}{\text{FR native}}\right) \times \left(\frac{\text{AB non-native}}{\text{AB native}}\right) \tag{2}$$

when RIP < 1, the predicted impact of the non-native fish is predicted to be less than the native; when RIP = 1, there is no difference in impact between the fish species; whereas when RIP > 1, the non-native has a greater impact than the native. To integrate uncertainty into the RIP score, a probability density function (pdf) was applied using the standard deviation (SD) of the FR and AB estimates and this generated 80% confidence intervals (CIs) (see Dick et al. 2017). Biplots were then generated to illustrate the RIP for both for non-native Mozambique Tilapia and Western Mosquitofish relative to the native River Goby at each season between ponds (Laverty et al. 2017). All analyses were carried out in R v. 3.4.2 (R Development Core Team 2017).

Results

Functional response

Prey survival of larval chironomids was 99% in control groups with predators absent, and thus prey mortality in the experimental groups was attributed to predation. Overall consumption was significantly different among fish species ($\chi^2 = 221.67$, $df = 2$, $p < 0.001$). Native River Goby consumed significantly fewer prey than both non-native Mozambique Tilapia ($z = 14.61$, $p < 0.001$) and non-native Western Mosquitofish ($z = 8.43$, $p < 0.001$). Mozambique Tilapia, in turn, consumed significantly more prey than Western Mosquitofish overall ($z = 6.41$, $p < 0.001$). Consumption was also significantly greater at the higher temperature, analogous with the summer season ($\chi^2 = 179.61$, $df = 1$, $p < 0.001$), and consumption increased with temperature for all species as there was no significant 'predator × temperature' interaction ($\chi^2 = 3.54$, $df = 2$, $p = 0.171$; Figure 1). Furthermore, consumption increased significantly with increasing prey supply ($\chi^2 = 2019.88$, $df = 1$, $p < 0.001$).

At 18 °C (i.e. spring temperature), all three fish species displayed a Type II functional response (Table 1; Figure 1a). Attack rates did not differ significantly between fishes (River Goby and Mozambique Tilapia: $z = 1.03$, $p = 0.301$; River Goby and Western Mosquitofish: $z = 0.42$, $p = 0.675$; Mozambique Tilapia and Western Mosquitofish: $z = 0.51$, $p = 0.611$). However, native gobies exhibited significantly longer handling times compared to both non-native Mozambique Tilapia ($z = 9.67$, $p < 0.001$) and non-native Western Mosquitofish ($z = 4.36$, $p < 0.001$). Accordingly, maximum feeding rates were considerably higher in the non-native as compared to native fishes (Table 1). In turn, Mozambique Tilapia had significantly shorter handling

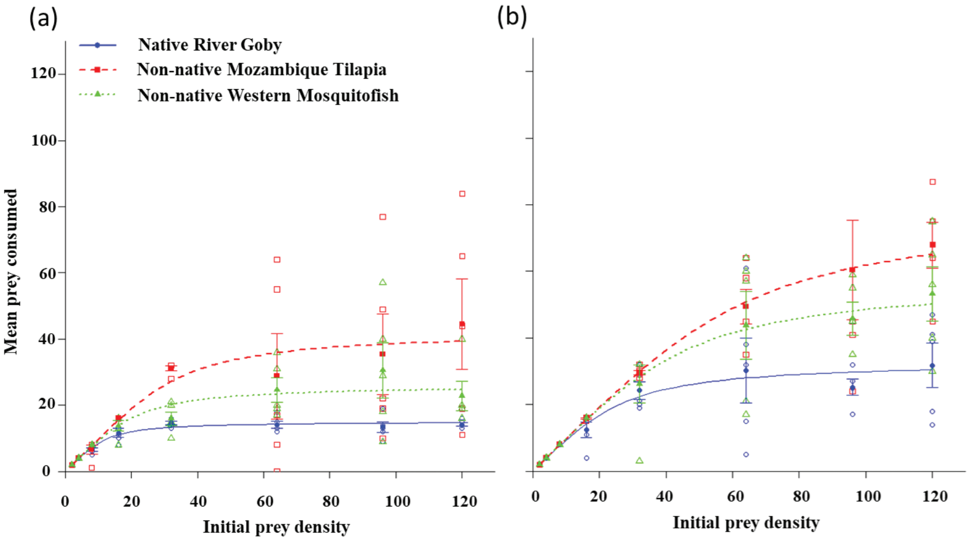

Figure 1. Functional response curves for native River Goby (blue circles, solid lines), non-native Mozambique Tilapia (red squares, dashed lines) and non-native Western Mosquitofish (green triangles, dotted lines) at 18 °C (a) and 25 °C (b). Means are ± SE. Filled points are means and unfilled points are raw data.

times, and thus higher maximum feeding rates, than Western Mosquitofish ($z = 6.27$, $p < 0.001$) (Figure 1a).

At 25 °C (i.e. summer temperature), all three fish species also exhibited a Type II functional response (Table 1; Figure 1b). There were significant differences in attack rates between the native River Goby and the non-native Mozambique Tilapia ($z = 2.62$, $p = 0.008$). Attack rates between the native River Goby and the non-native Western Mosquitofish did not differ significantly ($z = 0.24$, $p = 0.811$). However, Mozambique Tilapia had significantly lower attack rates than Western Mosquitofish ($z = 2.88$, $p = 0.004$). Native gobies displayed significantly longer handling times than non-native Mozambique Tilapia ($z = 12.55$, $p < 0.001$) and non-native Western Mosquitofish ($z = 7.18$, $p < 0.001$), again driving substantially higher maximum feeding rates by the non-native fishes (Table 1). In turn, Mozambique Tilapia had significantly shorter handling times than Western Mosquitofish ($z = 6.92$, $p < 0.001$), and hence exhibited the highest maximum feeding rate (Figure 1b).

Table 1. Parameter estimates from first-order logistic regression of the proportion of consumed prey as a function of prey density, with rounded functional response estimates, a = attack rate; h = handling time, $1/h$ = maximum feeding rate.

Predator	Temperature	First-order term, p	a	p	h	p	$1/h$
Native River Goby	18 °C	–0.04, <0.001	4.34	<0.001	0.05	<0.001	20.00
Non-native Mozambique Tilapia	18 °C	–0.03, <0.001	5.23	<0.001	0.02	<0.001	43.48
Non-native Western Mosquitofish	18 °C	–0.03, <0.001	4.74	<0.001	0.04	<0.001	27.78
Native River Goby	25 °C	–0.03, <0.001	3.65	<0.001	0.03	<0.001	34.48
Non-native Mozambique Tilapia	25 °C	–0.01, <0.001	2.20	<0.001	0.01	<0.001	111.11
Non-native Western Mosquitofish	25 °C	–0.02, <0.001	3.80	<0.001	0.02	<0.001	58.82

Fish abundances

There was a significant 'species × season × pond' interaction ($\chi^2 = 92.54$, $df = 2$, $p < 0.001$; Figure 2), with seasonal responses of fish species abundance differing between the two ponds. From ML Swart in spring, native River Goby abundances were not significantly different to non-native Mozambique Tilapia ($z = 0.63$, $p = 0.988$), but were more abundant than non-native Western Mosquitofish ($z = 4.44$, $p < 0.001$). In turn, the non-native Mozambique Tilapia were also more abundant than the non-native Western Mosquitofish ($z = 4.73$, $p < 0.001$). In summer, ML Swart abundances of the native gobies did not differ significantly either to non-native Mozambique Tilapia ($z = 0.48$, $p = 0.990$) or non-native Western Mosquitofish ($z = 2.71$, $p = 0.070$). In addition, there were no significant differences between non-native Mozambique Tilapia and non-native Western Mosquitofish abundances ($z = 2.23$, $p = 0.223$). On the other hand, from River Bend in spring, native gobies were significantly more abundant than both non-native Mozambique Tilapia ($z = 4.52$, $p = 0.001$) and non-native Western Mosquitofish ($z = 6.28$, $p < 0.001$). Non-native Mozambique Tilapia abundances were significantly greater than non-native Western Mosquitofish ($z = 3.51$, $p = 0.006$). In summer, however, gobies were significantly less abundant than non-native Mozambique Tilapia ($z = 10.74$, $p < 0.001$) yet more abundant than non-native Western Mosquitofish ($z = 4.12$, $p < 0.001$). Similarly, the non-native Mozambique Tilapia were more abundant than the non-native Western Mosquitofish here ($z = 5.74$, $p < 0.001$).

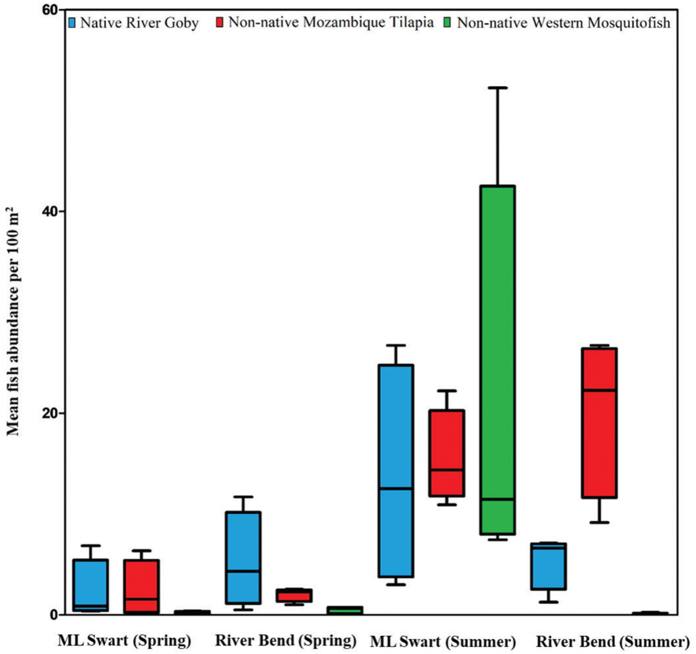

Figure 2. Abundance box plots for native River Goby (blue), non-native Mozambique Tilapia (red) and non-native Western Mosquitofish (green) from ML Swart and River Bend irrigation ponds, Eastern Cape, South Africa. Sampling occurred in spring (18 °C) and summer (25 °C).

Relative impact potential

Under both spring and summer treatments, the non-native Mozambique Tilapia consistently displayed relative impact potential scores > 1 relative to the native River Goby irrespective of focal ponds, suggesting greater impact than the native species (Table 2). In contrast, non-native Western Mosquitofish had relative impact potential scores of < 1 from ML Swart and approximately 1 from River Bend in spring, respectively suggesting lower or similar impacts to native River Goby (Table 2). In summer, non-native Western Mosquitofish had a relative impact potential score > 1 from ML Swart, but had a relative impact potential score < 1 from River Bend. This suggests less impact in River Bend and higher impact in ML Swart relative to native River Goby.

The relative impact potential biplots concur with the relative impact potential scores (Figure 3). In spring, non-native Mozambique Tilapia had the highest impact potential followed by native River Goby and lastly by non-native Western Mosquitofish in both ML Swart and River Bend (Figure 3a, b). In summer, there is inconsistency between the ponds, whereby the native River Goby has the lowest relative impact potential in ML Swart compared to the non-native Mozambique Tilapia and the non-native Western Mosquitofish (Figure 3c). The relative impact potential biplots from River Bend in summer are more reflective of the trends observed in both ponds in spring, where the non-native Mozambique tilapia had the highest impact potential followed by the native River Goby and lastly the non-native Western Mosquitofish, which had no impact owing to its absence here (Figure 3d).

Table 2. Relative Impact Potential (RIP) using mean bootstrapped maximum feeding rates for non-native Mozambique Tilapia and non-native Western Mosquitofish against native River Goby. Field abundance data are integrated from ML Swart and River Bend ponds in spring and summer. Uncertainties are reflected through 80% confidence intervals (CIs).

Species	Season	Pond	Mean FR maximum feeding ± SD	Mean field abundance ± SD	RIP	CIs	P_{rip}>1
Non-native Mozambique Tilapia, native River Goby	Spring	ML Swart	45.40 ± 11.31, 19.96 ± 3.53	2.41 ± 2.84, 2.25 ± 3.10	7.25	0.42 – 16.32	75.17
Non-native Mozambique Tilapia, native River Goby	Spring	River Bend	45.40 ± 11.31, 19.96 ± 3.53	2.04 ± 0.69, 5.22± 4.80	1.69	0.35 – 3.57	55.21
Non-native Western Mosquitofish, native River Goby	Spring	ML Swart	26.68 ± 2.87, 19.96 ± 3.53	0.19 ± 0.17, 2.25 ± 3.10	0.35	0.29 – 0.78	70.78
Non-native Western Mosquitofish, native River Goby	Spring	River Bend	26.68 ± 2.87, 19.96 ± 3.53	2.04 ± 0.69, 5.22 ± 4.80	1.01	0.22 – 2.11	33.40
Non-native Mozambique Tilapia, native River Goby	Summer	ML Swart	125.02 ± 54.57, 32.60 ± 4.10	15.50 ± 4.80, 13.70 ± 11.10	7.30	1.58 – 15.35	96.40
Non-native Mozambique Tilapia, native River Goby	Summer	River Bend	125.02 ± 54.57, 32.60 ± 4.10	20.10 ± 8.02, 5.41 ± 2.77	18.26	5.21 – 36.15	99.98
Non-native Western Mosquitofish, native River Goby	Summer	ML Swart	97.17 ± 148.60, 32.60 ± 4.10	20.70 ± 21.20, 13.70 ± 11.10	7.58	0.30 – 16.56	69.64
Non-native Western Mosquitofish, native River Goby	Summer	River Bend	97.17 ± 148.60, 32.60 ± 4.10	0.06 ± 0.13, 5.41 ± 2.77	0.05	0.00 – 0.09	40.20

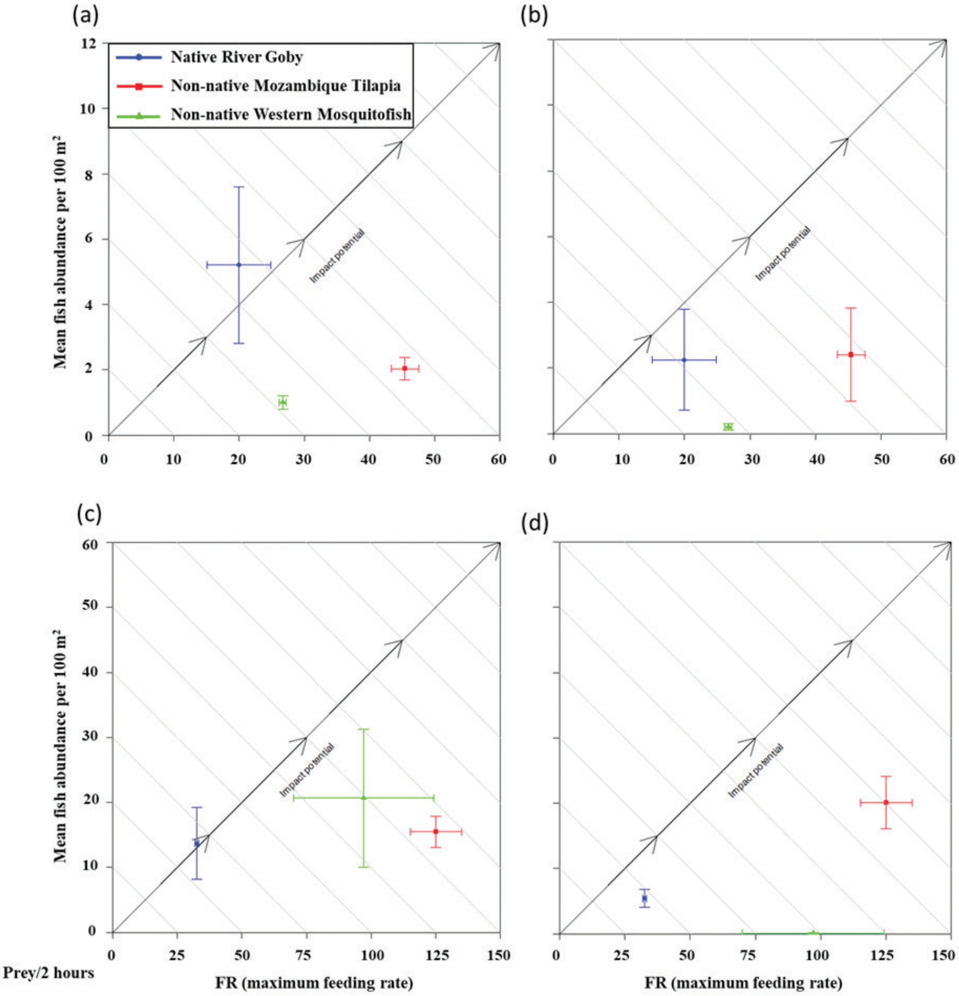

Figure 3. Relative impact potential (RIP) biplots (see also Table 2) of native River Goby (blue circles), non-native Mozambique Tilapia (red squares) and non-native Western Mosquitofish (green triangles) in spring (18 °C): (a) ML Swart (b) River Bend; and in summer (25 °C): (c) ML Swart and (d) River Bend. Ecological impact increases from bottom left to top right. Note differences in axes scaling. Values are mean ± SD.

Discussion

Using the relative impact potential metric proposed by Dick et al. (2017), this study provides insights into how the ecological impacts of non-native species are mediated by temporal variabilities associated with seasons through the multiplying of functional responses and population abundances. Irrespective of seasonal variations, our results corroborate with studies that identified Mozambique Tilapia as a particularly impactful non-native species (Canonico et al. 2005; Maddern et al. 2007), whilst Western Mosquitofish impacts were less pronounced. We first show that all three fish species display a Type II functional response across the seasonal gradient, conducive to high resource utilisation at low densities. Whilst Type II functional responses are common in comparative laboratory-based studies (e.g. Dick et al. 2013), if included experimentally, additional context-dependencies such as habitat structure may have driven a significant impact on functional response form (Vucic-Pestic et al. 2010a; Vucic-Pestic et al. 2010b; Kalinkat et al. 2013; Barrios-O'Neill et al. 2016). Moreover, greater incremental low-density prey resolution, different feeding durations and larger experimental aquaria volumes may further alter functional response forms (e.g. to Type III) (Sarnelle and Wilson 2008; Uiterwaal and DeLong 2018). Nevertheless, in the present comparative study, interspecific variation in functional responses between the species showed that both the non-native species exert higher *per capita* impacts than the native species on native prey and that predatory impacts are more profound during the summer season. These findings concur with a considerable number of studies comparing impact between invasive and native species (Alexander et al. 2014; Dick et al. 2014; Cuthbert et al. 2019).

Temperature differences had a significant effect on the functional response parameters, wherein attack rates were high in spring (i.e. 18 °C) and were reduced in summer (i.e. 25 °C). This result concurs with Grigaltchik et al. (2012), where an increase in temperature resulted in reduced attack rates, but contrasts with other studies (e.g. Wasserman et al. 2016) wherein attack rates exhibit a non-monotonic temperature response. Furthermore, we showed that during the summer season, handling times were reduced and hence these species exhibited higher maximum feeding rates. The findings from Englund et al. (2011) corroborate with ours, and this effect is mostly related to predators' metabolic rate changes. For instance, for a predator's metabolic activity to reach its maximum efficiency (i.e. high *per capita* effects), temperatures need to be optimal; yet if temperatures are too high this will result in reduced metabolic rates through catabolism (Clarke and Johnson 1999).

Secondly, we show that there was significant variation in fish abundances among species according to season, and also between ponds. Such variation in fish abundances seems to be a common theme, especially in fish communities that co-occur in environments and this is driven by spatial and temporal variation in life-history traits (Amezcua and Amezcua-Linares 2014). All three fish species were generally less abundant in spring and more abundant in summer. Mozambique Tilapia were the most abundant species overall, followed by River Goby and, lastly, Western Mosquitofish. By combining the fish maximum feeding rates and abundances (as per Dick et al. 2017) to give the relative impact potential score, we showed that the non-native Mozambique Tilapia consistently had the highest impact across seasons whereas, in the majority of cases, impacts of non-native Western Mosquitofish were less apparent relative to the native River Goby given currently low abundances.

Changes in relative impact potential scores with seasonal temperature fluctuations and fish abundances from different localities demonstrate how such context-dependencies can have a critical effect on the relative field impact capacities of introduced species through time (Dick et al. 2017). The effects of temperature regime shifts on interaction strengths are profound across habitat types and trophic groups (Englund et al. 2011; Rall et al. 2012), and increasing temperatures may exacerbate invader ecological impacts as species approach thermal optima (Iacarella et al. 2015). This is supported by the heightened functional responses observed for the non-native Mozambique Tilapia and non-native Western Mosquitofish as experimental temperature was increased to near their thermal preferendum (~28°C; Jobling 1981). Therefore, the explicit inclusion of temperature change will be critical in future studies which seek to predict invader impacts across regime shifts associated with climatic warming and seasonal variability. Since the relative impact potential metric was 100% predictive of ecological impact across taxonomic and trophic groups (Dick et al. 2017), the current results, whereby relative impact potential is high for the non-native Mozambique Tilapia, gives confidence that this species can be forecast to cause major ecological impacts.

The present study further demonstrates the usefulness of numerical response proxies such as abundances in rapid assessments of potential impacts of introduced species. Indeed, in many cases, impact predictions are inherently limited if based on *per capita* impacts alone, given the importance of abundances in discernments of overall offtake rates by consumer populations (Dick et al. 2017). Importantly, our results suggest that ecological impacts of non-native species are likely to change across seasonal gradients associated with both changing functional responses and abundances, with summer impacts generally more profound than those in spring. We thus propose that further studies should incorporate such seasonal variability. Our study demonstrates that species-specific shifts in abundances may alter interaction strengths within ecosystems towards native populations. Therefore, quantitative assessments of species abundances can ultimately bridge the gap in decision-making and can be used to forecast future invader impacts under different climatic conditions when combined with *per capita* effects. Nevertheless, our study additionally demonstrates that individual systems (e.g. ponds) can differ substantially in predator community composition over time, and this system-specific population variability should be also considered in future studies.

Overall, this study provides further evidence of the strength of the relative impact potential metric in predicting ecological impacts of species and provides an extension to the framework by integrating an environmental gradient, which reflects seasonal temperature fluctuations. The identification of temporal shifts in impact across seasons and habitats in our study presents novel insights into invader impact. In many ecosystems, data on species abundances are still lacking, but since the relative impact potential metric enables impact predictions for species without invasion histories, we recommend more surveys to estimate abundance of potential invaders and/or for practitioners to incorporate other proxies (such as fecundity) into the metric (see Dickey et al. 2018). Crucially, the ability of both Mozambique Tilapia and Western Mosquitofish to thrive in novel habitats highlights their ecological plasticity, and with an increase in environmental temperatures, their impacts may be intensified through changes to functional responses and fish abundances. The relative impact potential metric thus allows for rapid assessment of current and future invasive species under shifting environmental contexts and can identify priority species for management.

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Genetic and epigenetic regulation of phenotypic variation in invasive plants – linking research trends towards a unified framework

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Abstract

Phenotypic variation in the introduced range of an invasive species can be modified by genetic variation, environmental conditions and their interaction, as well as stochastic events like genetic drift. Recent studies found that epigenetic modifications may also contribute to phenotypic variation being independent of genetic changes. Despite gaining profound ecological insights from empirical studies, understanding the relative contributions of these molecular mechanisms behind phenotypic variation has received little attention for invasive plant species in particular.

This review therefore aimed at summarizing and synthesizing information on the genetic and epigenetic basis of phenotypic variation of alien invasive plants in the introduced range and their evolutionary consequences. Transgenerational inheritance of epigenetic modifications was highlighted focusing on its influence on microevolution of the invasive plant species. We presented a comprehensive account of epigenetic regulation of phenotypic variation and its role in plant invasion in the presence of reduced standing genetic variation, inbreeding depression and associated genomic events which have often been observed during introduction and range expansion of an invasive alien species. Finally, taking clues from the studies conducted so far, we proposed a unified framework of future experimental approaches to understand ecological and evolutionary aspects of phenotypic variation. This holistic approach, being aligned to the invasion process in particular (introduction-establishment-spread), was intended to understand the molecular mechanisms of phenotypic variation of an invasive species in its introduced range and to disentangle the effects of standing genetic variation and epigenetic regulation of phenotypic variation.

Keywords

epigenetics, evolution, genetic diversity, phenotypic variation, plant invasion, transgenerational inheritance

Introduction

With the increasing number of reports on negative impacts of invasive species on regional biota (Bellard et al. 2017; Early et al. 2016; Iacarella et al. 2015), biological invasion has become a severe problem globally and for obvious reasons, is in the spotlight of recent research trends. Numerous studies have been conducted to identify how a minor component of native communities has successfully established itself in a new and heterogeneous environment and becomes dominant in the invaded communities (Callaway and Maron 2006).

Multiple hypotheses have been put forward to explain successful invasion, e.g. resource fluctuation, enemy release hypothesis, evolution of increased competitive ability (EICA) [reviewed by (Catford et al. 2009; Inderjit et al. 2005)]. Among these, the influence of genetic diversity on invasion success has been long recognized (Baker and Stebbins 1965) and numerous studies have been conducted to explain this relationship [e.g. (Facon et al. 2006; Lavergne and Molofsky 2007; Roman and Darling 2007)]. Standing genetic variation can result in rapid selection of population showing greater fitness leading to successful establishment and range expansion of the introduced species (Barrett 2015; Sakai et al. 2001). On the other hand, introduction in a new location and range expansion from the point of introduction may cause population size reduction (demographic bottleneck) which can reduce genetic variation (Estoup et al. 2016; Uller and Leimu 2011), and subsequently may lead to inbreeding and considerable loss of fitness (Schrieber and Lachmuth 2017). However, even genetically depleted founder populations may establish and spread successfully if:

i) detrimental inbreeding effects are mitigated (Hufbauer et al. 2013; Rosche et al. 2017; Schrieber and Lachmuth 2017) by one or several of preventive mechanisms like multiple introductions [(Dlugosch and Parker 2008a; Uller and Leimu 2011); but see Hagenblad et al. 2015], genetic admixture among introductions (Roman and Darling 2007), preadaptation to the environment found in the introduced range (Hufbauer et al. 2012), and polyploidy (Pérez et al. 2006), or

ii) the plasticity of ecologically relevant traits of a genotype is enhanced in a way it can take advantage of a wider ecological niche (Bossdorf et al. 2008; Muth and Pigliucci 2007; Richards 2006; Spens and Douhovnikoff 2016; Walls 2010). Evolutionary changes in traits related to log-distance dispersal, growth rate, tolerance to environmental heterogeneity, and competitive ability in response to novel environmental conditions have been found to promote invasiveness (Bhattarai et al. 2017; Davidson et al. 2011; van Kleunen et al. 2010).

Epigenetic modifications in gene expression, being independent of any changes in DNA sequence (Nicotra et al. 2010; Richards 2006; 2011; Scoville et al. 2011), have been recognized as key mechanisms behind the expression of inbreeding depression (Biémont 2010; Nebert et al. 2010; Vergeer et al. 2012) and plastic responses of plant traits to environmental cues (Herrera and Bazaga 2013). Epigenetic changes can be induced by environmental stresses, both biotic and abiotic [e.g. (Dowen et al. 2012; Verhoeven et al. 2010)], and invasive plants are frequently exposed to these interactions in their introduced environment (Blackburn et al. 2011; Nunez-Mir et al. 2017; Zefferman et al. 2015). However, while there has been some progress in epigenetic studies in model and non-model organisms, studies with invasive plants have so far been limited to reviewing broad patterns of epigenetic variation (Richards et al. 2017). Moreover, it has been found that adaptive evolution to local conditions, phenotypic plasticity, or sometimes a combination of both, help invasive species to compete in a range of environments (Liao et al. 2016; Montesinos and Callaway 2018). Experimental studies on local adaptation and phenotypic plasticity are often conducted separately (but see Liao et al. 2016), thereby leaving a gap in comprehensive understanding of relative contribution of genetic differentiation and epigenetically regulated phenotypic variation on invasion success.

In this context, a comprehensive appraisal of the role of genetic and epigenetic variation in plant invasion and future prospects for investigation appears to be timely. This review was therefore framed to i) recognize the factors responsible for phenotypic variation; ii) identify the role of epigenetic processes in maintaining fitness of invasive plants; and iii) to propose a unified framework of experimental approaches to understand the relative importance of genetic differentiation and epigenetic regulation of trait fitness.

Factors responsible for phenotypic variation in the introduced range

Genetic basis of phenotypic variation

In the first step of the invasion process, a species can be introduced from its native range either by introduction of a few or even only a single genotype or through multiple introductions from different source populations of its native range. Multiple introductions of the species may give rise to two situations: i) the introduced genotype(s) can be restricted within the introduced region(s) and/or ii) multiple introductions from different source populations, breaching of geographical barriers, intra- or interspecific hybridization may produce genetically diverse populations and different phenotypes (phenotypic divergence). Phenotypic variation among the introduced populations is therefore dependent on the number of introduced genotypes (standing genetic variation) and can be increased by intra- and inter-specific hybridization. In addition to standing genetic variation, new mutations may also contribute to phenotypic variation (Fierst 2011; Lambertini et al. 2010). Recent studies found that a variety of mutation types occur frequently in the founding populations and these structural as well as regulatory mutations can have large effects on phenotype (Dlugosch et al. 2015). Given the short time frame for the introduced populations to respond to selection forces, standing genetic variation may contribute largely for adaptive evolution (Prentis et al. 2008). However, the arrival of new mutations may also provide scopes of selection of traits at low effective population sizes during range expansion of introduced species (Dlugosch et al. 2015). Moreover, phenotypic divergence in the introduced range often occurs under non-equilibrium demographic conditions and is frequently affected by prior evolutionary history in native range and stochastic events (e.g. genetic drift) (Keller and Taylor 2008). Natural selection can, therefore, act in native as well as in invasive range or during establishment in the invasive range (adaptive divergence).

Epigenetic basis of phenotypic variation

In addition to genetic-differentiation driven phenotypic divergence among the introduced populations, an individual genotype may also produce phenotypic variation in response to different environmental conditions of the introduced range (phenotypic plasticity). Epigenetic changes (without any change in DNA sequence) can contribute to phenotypic variation in plant traits independently of genetic variation (Richards et al. 2012). Therefore, while in the case of genetically diverse populations, both local adaptation and phenotypic plasticity may contribute to successful establishment of an invasive species in a novel environment, epigenetically regulated phenotypic variation may be responsible for the establishment of an invasive population in the absence of genetic variation. Epigenetic responses are caused by reversible enzyme mediated modifications of DNA, associated histones, and the generation of regulatory small non-coding RNA molecules leading to controlled transcriptional activity of genes, repetitive sequences and transposable elements (TEs) (Pikaard and Mittelsten Scheid 2014). These epigenetic variations can be induced by several developmental signals and environmental stresses (Chinnusamy and Zhu 2009). One of the best studied epigenetic mechanisms to date in plants is DNA methylation in which a methyl group is added to one of the four bases (usually cytosine) in the DNA molecule (Finnegan et al. 1998). Cytosine methylation occurs in CG, CHG and CHH contexts, where H = Adenine (A), Cytosine (C) or Thymine (T) nucleotides, and the reaction is catalyzed by methyltransferase enzyme. DNA methylation is enzymatically reversible by the action of DNA glycosylase enzymes.

Several studies have been conducted on model and non-model species, both in field and controlled conditions to quantify epigenetic influence on trait variation being independent of genetic variation (Abratowska et al. 2012; Latzel et al. 2013; Wu et al. 2013). For example, in a controlled greenhouse study and reciprocal transplant experiment, genetic and epigenetic diversity were compared across 16 populations of Japanese knotweed (*Fallopia japonica*) from three habitat types of its invaded range in USA (Richards et al. 2012). This study found higher epigenetic variation across habitat types, response of some epigenetic loci to local microhabitat conditions and low genetic diversity across populations. Comparing populations of *Poa annua* from its Antarctic (introduced) and Polish (native) ranges, Chwedorzewska and Bednarek (2012) found lower genetic differentiation but increased epigenetic variation in the introduced range compared to the native populations. These evidences indicated that epigenetic variation can contribute to phenotypic variation in plant traits independently of genetic variation. Epigenetic variation can be operational even on a short time scale as evident from a study on an invasive plant *Alternanthera philoxeroides*. Epigenetic variation in morphological traits of this invasive plant was examined in two habitats (aquatic and terrestrial), first in natural condition followed by common garden experiments (Gao et al. 2010). Considerable DNA methylation polymorphisms were observed within and between natural populations. Reciprocal transplantation of the ramets from the source populations induced morphological changes and epigenetic reprogramming, thereby indicating reversible induction of DNA methylation in a short period of time.

In addition to environmentally induced epigenetic variation, spontaneous epimutation may also cause the observed epigenetic differences among natural population. For example, a multi-generation common garden experiment on *Alternanthera philoxeroides* revealed that a combination of environmental induction and spontaneous epimutation resulted in epigenetic variation in the species (Shi et al. 2019). These epigenetic variations, either induced environmentally or resulting from spontaneous epimutation or both, may be stably inherited across generations (Jablonka and Raz 2009). This phenomenon is usually termed as transgenerational epigenetic inheritance and in plants, this process depends on a methyltransferase enzyme that replicates methylation patterns during both mitosis and meiosis (Takeda and Paszkowski 2006). In case of sexual reproduction, either meiotic resetting of epigenetic variation may occur or the epigenetic changes may bypass the surveillance mechanisms and are transmitted to the next generation. In clonal propagation, epigenetic changes are more stably inherited to the progeny since it is assumed that meiosis does not occur in vegetative reproduction. These heritable epigenetic modifications provide a platform for natural selection to act on ecologically relevant traits (Prentis et al. 2008), thereby contributing to the microevolution of natural populations (Bossdorf et al. 2008; Richards et al. 2017). Two mechanisms have been proposed to explain the role of epigenetic modifications in the evolution of natural populations.

First, similar to genetic variation, heritable epigenetic variation may translate into phenotypic variation and fitness differences among individuals for natural selection to act on. On the other hand, unlike genetic variation, epigenetic variation is altered by environmental conditions directly and, therefore, may provide an additional, accelerated way for evolution (Bossdorf et al. 2008). For example, population genomic analysis of three climatologically distinct *Quercus lobata* populations (Platt et al. 2015) revealed that DNA methylation (specifically, CpG methyl polymorphisms) was involved in local adaptation, either directly or through linkage to regions under selection.

Secondly, epigenetic mechanisms play a role in adaptive transgenerational plasticity, defined as the ability of the parent population to alter traits in their offspring which may enhance their fitness in similar environmental conditions (Galloway and Etterson 2007). Unlike mammals in which resetting of DNA methylation takes place during early embryonic development (Santos et al. 2002), the epigenetically induced phenotypic changes in plants can be inherited over several generations (Bräutigam et al. 2013) and thus give rise to epialleles (Jablonka and Raz 2009; Schulz et al. 2014). Epialleles can be defined as the forms of a gene that are responsible for heritable phenotypic variation without changing DNA sequence (Quadrana and Colot 2016). The best studied examples so far highlight the inheritance of induced epigenetic effects to the unstressed progeny of parents exposed to biotic and abiotic stresses (reviewed by (Holeski et al. 2012)). For example, in an experiment with multiple genetic lines of annual *Polygonum persicaria*, parental plants were grown in dry (drought-stressed) versus moist (well-watered) soil and their offspring were exposed to a demethylating agent zebularine during seed germination (Herman and Sultan 2016). Under controlled conditions (without zebularine treatment), the offspring of dry soil grown (drought-stressed) parental population produced longer root systems and more biomass in comparison to the offspring of moist soil grown parental population. Treatment with zebularine removed these developmental effects from the offspring of drought-stressed parents, while the offspring of well-watered parents showed non-significant alteration of phenotypic expression. These findings provide empirical evidence of epigenetic contribution to adaptive transgenerational plasticity from stressed parental population to offspring. However, the magnitude of epigenetic changes and their heritability may vary depending on the environmental conditions. For example, genetically identical apomictic *Taraxacum officinale* plants were exposed to different ecological stresses (salt, nutrient, chemicals mimicking herbivore and pathogen attacks) and the progeny of the stressed plants were raised in a common unstressed environment (Verhoeven et al. 2010). The study revealed heritability of induced changes; however, the variation in methylation pattern was noted among different stresses.

While most of the molecular investigations on transgenerational inheritance of epigenetic changes have been restricted to model and endemic species (Hauser et al. 2011; Henderson and Jacobsen 2007), evidences are rare for invasive plants which have been frequently exposed to biotic and abiotic stresses in the introduced environment. Exceptions exist, for example, in case of invasive *Fallopia* spp. (Japanese knotweed), Richards et al. (2012) observed epigenetic variation in leaves of the progeny plants after growing the rhizomes (collected from different habitats, *i.e.* across an abiotic stress gradient) in a common environment. This multigenerational experiment showed that parental exposure to abiotic stresses resulted in modified DNA-methylation in unexposed offspring.

Role of epigenetic processes in plant invasion

Genetic adaptation paradox and epigenetic regulation of phenotypic variation

After successful introduction (*i.e.* crossing the geographic and cultivation barriers, (Blackburn et al. 2011), some alien plant species establish wild populations in novel habitats. Two hypotheses, namely the ecotype hypothesis and the plasticity hypothesis, have been proposed for invasive plants to explain this ability of habitat accommodation (Geng et al. 2007). The ecotype hypothesis suggests that genetically based variations leading to local adaptation are responsible for thriving across different habitats. The positive relationship between genetic diversity of the founder population and invasion success (in terms of higher population growth rates and higher adaptability and dispersal ability) has been well-established (Bock et al. 2015; Collins et al. 2018; Crawford and Whitney 2010). A meta-analysis of differences in the frequency and magnitude of local adaptation between 47 alien invasives and 91 native species showed that local adaptation in invasive plant species was frequent and comparable to that exhibited by native plant species (Oduor et al. 2016).

However, contrasting examples also exist where introduced plant populations with very low genetic diversity (and lower in comparison to native populations) have been found to be successful invaders (Hagenblad et al. 2015; Ren et al. 2005; Zimmermann et al. 2010). For example, a global scale population genetic survey using amplified fragment length polymorphism (AFLP) markers of the aquatic invader *Eichhornia crassipes* (water hyacinth) revealed very low genetic diversity in the introduced populations, 80% of which were composed of a single clone leading to little differentiation compared with those from the native range (Zhang et al. 2010). In a recent study on invasive *Fallopia* (Japanese knotweed) in Norway (northerly distribution range in Europe), no genetic variation was observed within this invasive taxon (Holm et al. 2017).

Populations with such restricted genetic variation may find other mechanisms to extend the ability of a single genotype, or general-purpose genotype or GPG (Baker 1965), to take advantage of a wider ecological niche (Spens and Douhovnikoff 2016). This paradox of invasion success of the introduced populations in spite of having low genetic diversity has been attributed to phenotypic plasticity of traits (plasticity hypothesis) (Bossdorf et al. 2005). Phenotypic plasticity is considered as one of the underlying mechanisms of general purpose genotype (GPG) model (Massicotte and Angers 2012) and is more important in rapidly fluctuating habitats (Clements and Ditommaso 2011). Many studies have highlighted the role of phenotypic plasticity on the successful invasion of exotic plant species (Hagenblad et al. 2015; van Kleunen et al. 2010), mostly for clonal species for which local adaptation is usually not observed (Geng et al. 2016). Clones of an invasive species *Alternanthera philoxeroides* showed varying levels of genetic diversity (in terms of both ISSR marker diversity and quantitative trait variation) between and within its native range (Argentina) and two invasive ranges - China and the USA (Geng et al. 2016). However, significant phenotypic plasticity in biomass allocation and morphological traits in response to varying water availability was observed in all clones regardless of their geographic origins, suggesting the possible role of phenotypic plasticity to invade diverse habitats across broad geographic areas. In addition, adaptive transgenerational plasticity contributes to the exotic species growth and successful establishment in a novel environment (Campbell et al. 2015; Dyer et al. 2010; Fenesi et al. 2014) and natural selection for particular traits may promote range expansion directly (Clements and Ditommaso 2011). For example, despite the loss of genetic variability in the invaded range, *Hypericum canariense* was found to be a successful invader in the Hawaiian Islands, San Diego and Calfornia, USA, and substantial adaptive evolution in growth rate and flowering phenology was found to overcome this genetic depletion (Dlugosch and Parker 2008b). Epigenetic modifications in gene expression and function have been recognized as key mechanisms behind phenotypic variation of plant traits in response to such environmental cues (Herrera and Bazaga 2013).

The potential role of epigenetics in the expression of inbreeding depression in founder populations

Reduced genetic diversity during invasions may not only result in a loss of adaptive potential; it may also increase inbreeding rates. Inbreeding enhances the phenotypic expression of deleterious recessive mutations leading to a loss of fitness in the offspring generation (*i.e.*, inbreeding depression), which can considerably hamper invasion success (Schrieber and Lachmuth 2017). Inbreeding depression is found more commonly in stressful environments (Reed et al. 2012) and multiple studies have been conducted to establish this synergistic relationship between inbreeding and environmental stress (e.g. (Campbell et al. 2013; Kariyat et al. 2012; Kristensen et al. 2010). Recent empirical studies support that inbreeding x environment interactions can prevent or foster successful invasion (Hufbauer et al. 2013; Rosche et al. 2017; Schrieber et al. 2019), while molecular studies suggest that epigenetic modifications play a decisive role in stress responses (Chinnusamy and Zhu 2009) and the expression of inbreeding depression. For example, a study on *Scabiosa columbaria* (a self-compatible but predominantly outcrossing species) revealed that inbreeding caused inbreeding depression for fitness-related traits and increased methylation levels (Vergeer et al. 2012). This study observed elimination of inbreeding depression by restoring the increased DNA methylation level in inbreds to the outbred level and concluded that DNA methylation could mediate the negative effects of inbreeding. In summary, these studies suggest that epigenetic changes may be involved in purging (i.e. recovery from inbreeding depression) (Nebert et al. 2010). However, to the best of our knowledge, empirical evidence on the relationship between epigenetic modifications and inbreeding depression in invasive plants is lacking. We require more basic knowledge on the role of epigenetics in the expression of inbreeding depression from the field of genetics and molecular biology before we can apply and test this concept in the context of plant invasions.

Epigenetic alterations associated with genomic events during plant invasions

Epigenetic modifications may not only contribute to establishing the success of genetically depleted plant founder populations, but they may also further enhance the adaptive potential of intra-or inter-specifically hybridized or polyploid invaders. Genomic events such as intra-or inter-specific hybridization between genetically distinct source populations and polyploid formation are responsible largely for speciation (Rapp and Wendel 2005) and increasing the evolutionary potential of invasive species (Rius and Darling 2014; van Kleunen et al. 2015) leading to successful invasion (Ellstrand and Schierenbeck 2000). Multiple introductions and intraspecific hybridization have been found to lead to the increase (or retention of) genetic diversity, and subsequently fitness of the invading population like *Bromus tectorum* (Novak and Mack 2005), *Phalaris arundinacea* (Lavergne and Molofsky 2007), *Senecio pterophorus* (Vilatersana et al. 2016), *Ambrosia artemisiifolia* (van Boheemen et al. 2017).

During these processes of intra-or inter-specific hybridization and allopolyploid formation, epigenetic alterations are found to be prevalent (Rapp and Wendel 2005). A classic example of epigenetic modification during intraspecific hybridization and its role in invasion success has been found in a series of studies involving the genus *Spartina* (Aïnouche et al. 2009; Parisod et al. 2009; Salmon et al. 2005). In these experiments, methylation repatterning was observed in two hybrid species (*Spartina* x *townsendii* and *Spartina* x *neyrautii*), although these hybrids were genetically uniform with their ancestors (American introduced *Spartina alterniflora* and European native *Spartina maritima*) (Salmon et al. 2005). These studies also identified intraspecific hybridization as a primary stimulus in the invasion success of polyploid *Spartina* species (Aïnouche et al. 2009). However, the connections between these epigenetic alterations and morphological or ecological phenotypes of the hybrids are yet to be discovered (Rapp and Wendel 2005). Nevertheless, genomic events (e.g. intra-or inter-specific hybridization, polyploidization) inducing epigenetic changes leading to morphological variation has been reported from various model plant systems, e.g. in allopolyploid *Brassica rapa* (diploid *Brassica napus* x *Brassica oleracea*) (Rapp and Wendel 2005). It is interesting to note that epigenetic modifications could vary between different groups of plants, and even between ploidy levels. For example, MS-AFLP analysis in synthetic *Gossypium* (cotton) tetraploids and hexaploids showed different methylation pattern in comparison to their diploid and tetraploid progenitors (Liu et al. 2001).

Towards a unifying research framework

One of the major objectives of this review has been finding a comprehensive structural guideline of experimental approaches taking clues from the studies already conducted on invasive and non-invasive, model and non-model species. Phenotypic variation in a plant species in its introduced range is one of the most highly-researched topics in invasion biology in which basic ecological research demonstrated the role of phenotypic variation in the invasion success of exotic species. On the other hand, genetic variation, microevolution and epigenetic processes have been found to play significant roles in the phenotypic variation of traits, and therefore, have been recognized as relevant to understand the mechanisms underlying the natural variation in ecologically important traits (e.g. Colautti and Barrett 2013; Liao et al. 2016; Marchini et al. 2019; Oduor et al. 2016). In this context, a bridge between these parallel but complementary experimental approaches may provide a comprehensive understanding of ecological and evolutionary aspects of phenotypic variation of traits and their roles in the invasion process (introduction-establishment-spread continuum). This empirical framework is, therefore, specifically aimed to broaden the scope of research by including the genetic investigation components into the ecological studies on the phenotypic variation of traits in the invasive species. For this purpose, a model system has been conceptualized based on an invasive plant species which has been reported to have i) phenotypic variation across environmental gradients, and ii) reproduction ability through both sexual and vegetative means. We first aligned the proposed framework with three different stages of invasion (introduction, establishment and spread) to identify the possible locations where genetic differentiation and/or epigenetic regulation can act (Figure 1). We proposed future experimental studies (Figure 2) to understand the relative importance of genetic and epigenetic regulation of trait fitness along the course of the invasion process. The methodologies usually adopted for these experiments have been given in Table 2 with their respective strengths and challenges while detailed methodologies for screening epigenetic variation in invasive plants have been provided in Box 1.

Field and controlled experiments are being conducted to characterize phenotypic variation of invading populations, often in comparison to their native congeners and to other species native to the invaded habitat (van Kleunen et al. 2010). Reciprocal transplant and/or common garden experiments are suitable for delineating the effects of local adaptation and phenotypic plasticity on successful invasion of an exotic species (Figure 2). In reciprocal transplant experiments, individuals from different populations are transplanted between the original habitats from where the populations were sampled and population x test habitat interactions are quantified in terms of fitness parameters (Kawecki and Ebert 2004). The alternative approach to this involves creating the properties of different habitats in greenhouse or experimental plots, where fitness functions of different populations have been quantified. This experimental set-up is known as common garden (explant) studies. In a common garden experiment, two or more populations of a species growing in their native and non-native environments are transplanted in a common environment so that the genetic basis of the observed differences among field populations can be identified (Molofsky et al. 2017; Parker et al. 2003). While in field experiments (marked '1' in Figure 2), identifying plastic responses to a set of well-defined stress factors is important for comparative studies (Gratani 2014), manipulation of resource conditions in a biologically meaningful manner is required in reciprocal transplant and/or common garden studies (marked '2.1' in Figure 2) to yield important and relevant information (Davidson et al. 2011). In a reciprocal transplant experiment, plants from different invasive populations can be grown in a common environment to compare fitness traits. Higher mean fitness for all the traits of the local population compared to foreign population will indicate local adaptation (Local versus Foreign comparison). Significant difference in trait values among transplant sites (for a population) will indicate plastic responses whereas difference among populations (for a site) will indicate genetic differentiation. However, this approach is often confounded by intrinsic issues of population quality, e.g., inbreeding and transgenerational effects (Blanquart et al. 2013). The average effects of transplantation can be measured by comparing trait values between the local site and all away sites (Sympatric versus Allopatric comparison); however, this approach has been also found to be confounded by strong local advantage.

For example, morphological differentiation was studied between weedy, non-native and non-weedy, native populations of *Centaurea solstitialis* in a common garden setting and further compared using neutral genetic variation at simple sequence repeat markers (Eriksen et al. 2012). This study found quantitative variation to be more strongly partitioned among regions than genetic variation, which suggests that local adaptation

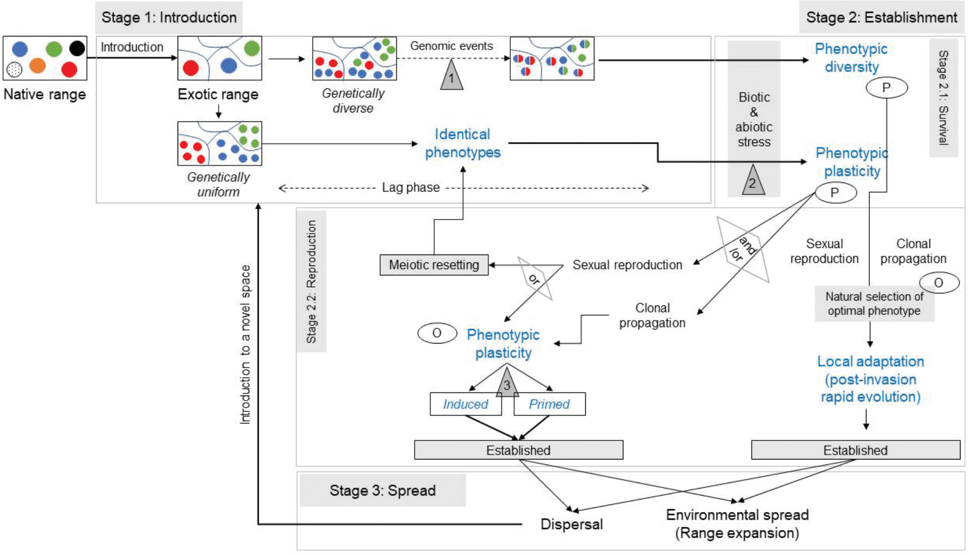

Figure 1. Conceptual framework for differentiating genetic and epigenetic basis for phenotypic variations across three stages of alien plant invasion process (introduction, establishment, spread). While genetic differentiation between introduced populations may cause phenotypic variation which leads to local adaptation and post-invasion rapid evolution through selection of traits and natural selection of optimal phenotype across environmental conditions, epigenetically regulated phenotypic variations are more prevalent in genetically similar populations. Three sites where epigenetic mechanisms may influence invasion success have been marked with triangles: 1) in case of genetic admixture between different genotypes present in a region, 2) biotic and abiotic stress induced epigenetic alterations among the genetically similar populations, and 3) transmission of epigenetic information from the parents (P) to the offspring (O) making the progeny capable of dealing with similar kinds of parental environment.

might play a role in successful invasion of the species. In a recent transplantation experiment, local adaptation and phenotypic plasticity were examined in terms of fitness responses for sexual and clonal reproductive measures and vegetative responses of an invasive plant *Fallopia japonica* across a broad latitudinal range within North America (van Wallendael et al. 2018). This study reported significant effects of the source population (suggesting genetic differentiation) and transplant sites (suggesting phenotypic plasticity) for all vegetative traits, but no evidence of local adaptation was found for sexual or clonal reproductive traits. Contrasting examples are also found in which phenotypic plasticity can be operational being independent of genetic variation. For example, trait plasticity and genetic variation were examined across 16 populations of Crofton weed (*Eupatorium adenophorum*) in China in a common garden experiment followed by intersimple sequence repeat (ISSR) marker analysis. This study revealed the presence of high phenotypic plasticity of functional traits despite having low genetically based variation (Zhao et al. 2012).

However, phenotypic differentiation in invading populations may also arise from random shifts in allele frequencies during repeated demographic disequilibrium (i.e.,

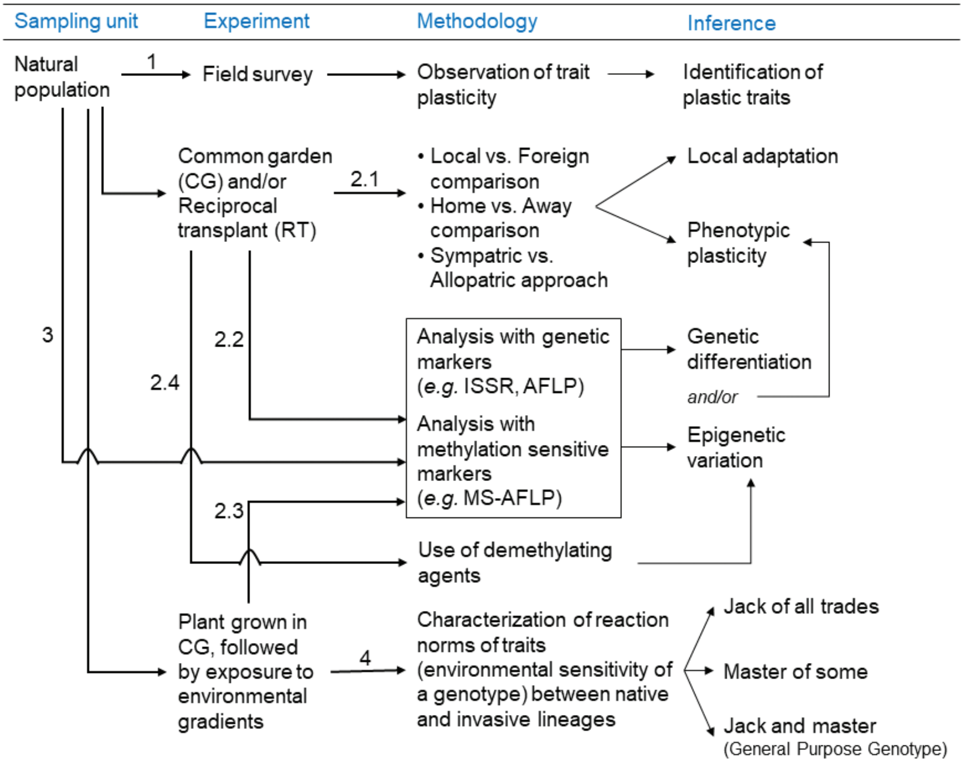

Figure 2. Experimental framework for differentiating genetic and epigenetic regulation of phenotypic plasticity. While a field survey of natural populations may identify plastic traits (1), reciprocal transplant experiments comparing performances of local and foreign populations may give insights into local adaptation, phenotypic plasticity and genetic differentiation as well (2.1). Plants grown in common garden experiments may be subjected to analysis with genetic and methylation-sensitive markers (2.2) or they can be exposed to environmental stresses before analysis (2.3) to identify genetic and epigenetic variation regulating trait plasticity. The use of demethylating agents (2.4) can also provide indirect evidence of transgenerational epigenetic inheritance. Samples from the natural population can also be analyzed with these markers followed by proper statistical analysis to disentangle genetic and epigenetic effects on trait plasticity (3). Characterization of reaction norms of the plants (e.g. comparison between native and invasive lineages) grown in common garden in response to environmental gradients (4) may highlight the trade-offs between maintaining a high performance across a range of conditions (robustness or jack of all trades) and maximizing fitness in an environmental condition (opportunism or master of some) or both (robust to environmental conditions and high performance, the general-purpose genotype).

genetic drift). Thus it is necessary to account for non-adaptive evolutionary change when investigating adaptive differentiation in invaders (Keller and Taylor 2008). In this context, information from neutral genetic markers can be used to control for, and quantify the effect of, non-adaptive processes with different statistical approaches (Agrawal et al. 2015; Keller et al. 2009; Meimberg et al. 2010; Schrieber et al. 2017). For example, genetic differentiation in phenotypic traits across environmental gradients was tested

Table 1. Examples of experimental studies investigating the role of epigenetic variation in phenotypic plasticity in both non-native and native, model and non-model species in controlled as well as field-based experiments. The factors which may influence the experimental designing and outcomes are mentioned here: species reproduction (sexual, vegetative, or both), plant material used, environmental gradient responsible for epigenetically controlled plastic changes and genetic as well as methylation sensitive genetic marker-based analysis (AFLP = Amplified Fragment Length Polymorphism; MS-AFLP/MSAP/met-AFLP = methylation sensitive AFLP).

Obs.	Name of the species	Species status	Species reproduction	Experimental design	Plant material	Environmental gradient	Methodology	Reference
1	*Fallopia* sp. (Japanese knotweed)	Invasive	Vegetative and sexual	Controlled	Rhizome – Leaf	Diverse habitats	AFLP and MS-AFLP	Richards et al. (2012)
2	*Poa annua*	Non-native	Sexual	Field based	Shoot	Comparison between native & invasive populations	AFLP and met-AFLP	Chwedorzewska and Bednarek (2012)
3	*Alternanthera philoxeroides*	Non-native	Vegetative	Field based Common garden	Leaf Plant	Habitat – Aquatic and terrestrial	AFLP and MSAP	Gao et al. (2010)
4	*Spartina* sp. (5 species – 2 parents, 2 hybrids and 1 allopolyploid	Non-native	Sexual	Controlled	Leaf	Allopolyploid speciation	AFLP and MSAP	Salmon et al. (2005)
5	*Phragmites australis*	Introduced invasive and native non-invasive subspecies	Facultative clonal	Field based	Leaf	Comparison between native & invasive subspecies	AFLP and MS-AFLP	Spens and Douhovnikoff (2016)
6	*Ageratina adenophora* (Crofton Weed)	Non-native	Sexual and vegetative	Controlled	Leaf	Cold tolerance	*ICE1* gene methylation	Xie et al. (2015)
7	*Taraxacum officinale*	Endemic	Apomictic	Controlled	Seed – Leaf	Nutrient, Salt, Pathogen attack	AFLP and MS-AFLP analysis	Verhoeven et al. (2010)
8	*Arabidopsis thaliana*	Model species		Controlled	Seed – Leaf		Demethylating agent 5-azacytidine	Bossdorf et al. (2010)
9	*Viola cazorlensis*	Endemic	Sexual	Field based	Leaf	Adaptive epigenetic variation	AFLP and MSAP	Herrera and Bazaga (2010)
10	*Viola elatior*	Endemic	Vegetative and sexual	Field based	Leaf	Light availability	AFLP and MSAP	Schulz et al. (2014)
11	*Betula ermanii*	Endemic	Sexual	Field based	Leaf	Habitat	AFLP and MS-AFLP	Wu et al. (2013)
12	*Armeria maritima*	Endemic	Obligatory outbreeding	Controlled	Seed – Leaf	Metal concentration	AFLP and met-AFLP	Abratowska et al. (2012)
13	*Ilex aquifolium*	Endemic	Sexual	Field based	Leaf – heterophylly	Herbivory	MSAP	Herrera and Bazaga (2013)
14	*Laguncularia racemose*	Mangrove-endemic	Vegetative and sexual	Field based	Leaf	Habitat	MSAP	Lira-Medeiros et al. (2010)
15	*Viola cazorlensis*	Endemic	Sexual	Field based (long term: 20 years)	Leaf	Herbivory	AFLP and MSAP	Herrera and Bazaga (2011)

Based on a literature search on Web of Science to find experimental studies conducted to establish relationship between epigenetic variation and phenotypic plasticity. We used the search phrase "("phenotypic plasticity" AND "plant") AND ("epigenetic") AND ("transgenerational plasticity") AND ("epigenetics" OR "methylation"). The search result yielded 30 papers from which the empirical studies have been summarized in this table. We also considered references cited in these papers and the experimental studies have been included in this table. The references of these studies have been cited in the literature section.

between native and introduced populations of two perennial plants *Silene vulgaris* and *S. latifolia* in a common garden experiment (Keller et al. 2009) using AFLP loci and statistically controlling neutral processes like colonization history, gene flow and genetic drift. The results revealed evidence of adaptive differentiation for some traits while the role of neutral processes governing phenotypic variation was also found for other traits.

To identify genetic and epigenetic regulation of phenotypic variation, the invasive populations of the common greenhouse environment can be subjected to analysis with genetic and methylation-sensitive markers (marked 2.2 in Figure 2) [e.g. Richards et al. (2012)]. The plants can be exposed to environmental stresses in common garden experimental set-up and stress-induced phenotypic variation can be analyzed using both genetic and methylation-sensitive markers (marked 2.3 in Figure 2) (2.3) (e.g. Verhoeven et al. 2010). Samples from natural populations can also be analyzed with these markers followed by proper statistical analysis to disentangle genetic and epigenetic effects on trait variation (3) (e.g. Herrera and Bazaga 2013). In addition, use of demethylating agents like 5-azacytidine and zebularine inhibits the enzyme methyltransferase activity of DNA demethylation and therefore natural epigenetic variation can be identified from responses of different natural populations to the treatment of these demethylating agents (marked 2.4 in Figure 2). Similar approaches have been found successful to identify genetic and epigenetic regulation of phenotypic variation in model and non-model species (Table 2). For example, a set of natural genotypes of the model species *Arabidopsis thaliana* was treated with demethylating agent 5-azacytidine, and the effect of reduced DNA methylation was identified as the main cause of the observed phenotypic changes of plant traits (Bossdorf et al. 2010). In another greenhouse experiment, individuals of six genotypes of a perennial grass species *Festuca rubra* were treated with 5-azacytidine and their performances were measured across different environmental conditions (Münzbergová et al. 2019). This study found interactive effect of demethylation with the environment and genotype, thereby suggesting that epigenetic variation can be influenced by both genetic structure and local environment.

Experiments involving multiple generations of the species may detect the heritability of plastic traits across generations (stage 2.2 in Figure 1). The progeny population can be grown from the seeds or the clonal fragments of the parental population in the common garden experiment, and trait variability can thereafter be analyzed using genetic and methylation-sensitive markers. Alternatively, demethylating agents can also be used to have indirect evidence of transgenerational epigenetic inheritance. While the majority of these studies have been conducted across sexual generations [(e.g. (Herman and Sultan 2016)), very few studies identify adaptive transgenerational effects in clonally reproducing plant species, although clonal reproduction is recognized as the main reproductive strategy for most plant species (Rendina González et al. 2018). For example, adaptive transgenerational effects in clonal offspring of *Trifolium repens* were tested after exposing parental generation to drought and herbivory stress (Rendina González et al. 2017). 5-azacytidine was used to decrease the global methylation level of DNA relative to control plants. The study found an increased number and size of offspring ramets (branches arising from the transplanted stolon) from the parents

Table 2. Experimental designs commonly used for investigating the effect of epigenetic variation on phenotypic plasticity and transgenerational pattern of epigenetic changes across generations. Strengths and challenges associated with each of these approaches have been mentioned.

Experimental design		**Examples**	**Strengths**	**Challenges**
Study system	**Study procedure**			
Natural population	1. Sampling from plant materials (leaf, shoot) of identical developmental stages across a disturbance gradient 2. Analysis with molecular markers and methylation sensitive restriction enzymes (Box 1) 3. Statistical analysis to identify epigenetic variation that is not explained by genetic variation	(Herrera and Bazaga 2013; Schulz et al. 2014)	1. Consider dynamic ecological factors that exist in wild populations (Spens and Douhovnikoff 2016) 2. Three-way relationship (environment x phenotypic plasticity x epigenetic changes) can be established	1. Cannot identify whether the observed differences reflect heritable variation or repeated introduction (Richards et al. 2017) 2. Challenging for sexually reproducing organisms in which genetic and epigenetic variation may be closely intertwined (Herrera and Bazaga 2013)
Controlled experiments				
Common garden – I	1. Sampling of reproductive materials (rhizomes, seeds) from the field population across a disturbance gradient 2. Grow materials in a common environment 3. Sampling from plants grown in the controlled environment 4. Analysis with molecular markers and methylation sensitive restriction enzymes (Box 1) 5. Statistical analysis to identify epigenetic variation that is not explained by genetic variation	(Abratowska et al. 2012; Richards et al. 2012)	1. Minimization of epigenetic differences induced among sampling locations 2. Detection of stable and heritable (through clonal propagation) epigenetic changes (Bossdorf et al. 2008) 3. By controlling genetics and environment, quantification of epigenetic variation is possible	1. Experimental design may be narrow and therefore, may oversimplify the dynamic ecological factors existing in the wild populations (Spens and Douhovnikoff 2016) 2. Not suitable for outcrossing species as genetic identity of the field population is unknown
Common garden – II	1. Collection of known genotypes (e.g. from seed stocks, seeds from asexual variants of apomictic plants) 2. Exposure to environmental treatments 3. Seeds collected from treated plants and grown in control environment 4. Samples from controlled environment plants 5. Analysis with molecular markers and methylation sensitive restriction enzymes (Box 1)	(Bossdorf et al. 2010; Verhoeven et al. 2010)	1. Identification of stress induced DNA methylation patterns 2. Heritability of traits	Not suitable for sexually reproducing species in case the genetic variation is unknown and seed stock is not available
Natural population + Common garden	1. Genetic and epigenetic profiling (Box 1) from field sampled plant materials 2. Grow material in a common environment 3. Reciprocal transplantation of the plants grown in common environment 4. Sampling from the transplanted plants 5. Analysis with molecular markers and methylation sensitive restriction enzymes (Box 1) 6. Statistical analysis to identify epigenetic variation that is not explained by genetic variation	(Gao et al. 2010)	1. Identification of epigenetic changes at a temporal scale (a plant's life time) 2. Direction of epigenetic alteration (reversible)	Challenging for sexually reproducing plant species

Box 1. Methodologies for screening epigenetic variation in invasive plants.

Molecular markers with methylation sensitive restriction enzymes:

A standard Amplified Fragment Length Polymorphism (AFLP) process followed by methylation sensitive AFLP (MS-AFLP or MSAP). In MS-AFLP, pairs of methylation sensitive restriction enzymes (isoschizomers) have been used to survey cystine methylation at restriction sites spread across the investigated genomes. In AFLP, MseI and EcoRI have been used to digestion of DNA extracts whereas HpaII and MspI with EcoRI have been used in MS-AFLP. AFLP and MS-AFLP are usually applied in parallel to compare genetic and epigenetic structures of populations using statistical techniques. Unlike HPLC- and ELISA-based assays which determined the proportion of methylated cytosines across the entire genome, the MS-AFLP can distinguish between different genomic locations or contexts (CG, CHG, CHH) of cytosine methylation from the banding patterns: CpG methylated loci (bands present in EcoRI/MspI only); nonmethylated loci (bands present in both profiles); loci hemimethylated at the external C of the restriction site (bands present in EcoRI/HpaII only) and noninformative loci (bands absent in both profiles). This methodology has been successfully applied to screen epigenetic variation in both invasive species [e.g. *Alternanthera philoxeroides* (Gao et al. 2010); *Fallopia* sp. (Iacarella et al. 2015; Richards et al. 2012); *Phragmites australis* (Spens and Douhovnikoff 2016)] and non-invasive species [*Taraxacum officinale* (Verhoeven et al. 2010); *Viola elatior* (Catford et al. 2009; Roman and Darling 2007; Schulz et al. 2014)].

Although the commonly used pair of isoschizomers (HpaII/MspI) can identify changes in methylation pattern, they fail to support data concerning genetic variation exclusively. To circumvent this limitation, some authors suggested use of met-AFLP along with AFLP procedure. In met-AFLP, the restriction enzymes Acc65I/MseI and KpnI/MseI have been used. For example, Chwedorzewska and Bednarek (2012) used AFLP and met-AFLP to characterize genetic and epigenetic variation in invasive *Poa annua* population in Antarctica. In case of non-invasive species, Abratowska et al. (2012) used this procedure to identify genetic distinctiveness of metallicolous and non-metallicolous populations of a metallophyte, *Armeria maritima*.

Future directions:

Among the advanced and more powerful technologies, bisulfite sequencing-based methods are now being used for screening epigenetic variation (e.g. Schield et al. 2016; Spens and Douhovnikoff 2016; van Gurp et al. 2016). In these methods, unmethylated cytosines are converted to uracil, and methylated cytosines are identified by comparing a treated sample to a reference sample. Quantification of small (s) RNAs that influence de novo establishment and maintenance of DNA methylation at many sites may also provide insights into the heritable epigenetic variation in plants (see Bond and Baulcombe 2014).

grown in drought condition and increased growth of offspring ramets from the parents treated with repeated application of jasmonic acid. Application of 5-azacytidine to the parents exposed to the drought condition and application of jasmonic acid reduced the growth of maternal ramets (transplanted main stolon). These findings provide evidence that parental environment can induce transgenerational effects in the offspring and some of these effects can be adaptive.

Focusing on a specific gene methylation variation can also provide two important insights: in case of genetically uniform species, variation in gene or protein expression (measured using microarrays or 2-D electrophoresis) indicate underlying epigenetic variation (Bossdorf et al. 2008) and secondly, the expression of the methylated gene may highlight the mechanism by which methylation differentiation contributes to the successful invasion (Xie et al. 2015). For example, the C-repeat/dehydration-responsive element binding factor (CBF) pathway governs plant responses to adverse low temperature (Chinnusamy et al. 2003). Demethylated upregulation of cold response regulator *ICE1* (inducer of CBF expression 1) was found to be the evolutionary mechanism responsible for northward expansion of the invasive *Ageratina adenophora* (Crofton weed) in China (Xie et al. 2015). Use of Quantitative Trait Loci (QTL)-mapping approaches can be useful to link the natural epigenetic variation with the observed phenotype. QTLs have been recognized as genetic regions (associated with phenotypic traits) which control the magnitude of a specific trait (Cortijo et al. 2014). Epigenetic QTLs, or the loci associated with different methylation marks, have been found to control flowering time and root length in the model plant *Arabidopsis*, thereby demonstrating that heritability of some traits can be determined by epigenetic variation (Cortijo et al. 2014).

Finally, a higher degree of phenotypic plasticity in an invasive species does not necessarily mean that the species has become invasive due to the plasticity (Palacio-López et al. 2015). To infer the role of phenotypic plasticity in successful invasion, observations of trait plasticity should be followed by experimental studies to identify that the plastic response is adaptive or linked to fitness (Davidson et al. 2011; van Kleunen and Fischer 2005). Characterization of reaction norms of the study species (e.g. comparison between native and invasive lineages) grown in common garden in response to environmental gradients (marked ‘4’ in Figure 2) may highlight the trade-offs between maintaining a high performance across a range of conditions (robustness or jack of all trades) and maximizing fitness in an environmental condition (opportunism or master of some) or both (robust to environmental conditions and high performance, the general purpose genotype) (e.g. Drown et al. 2011).

Concluding remarks

This review, being especially focused on plant invasion, has provided a comprehensive account of the molecular mechanisms of trait fitness of invasive plants. The strength of this review lies in the proposed framework that will encapsulate the ecological and evolutionary aspects of phenotypic variation. Future ecological studies should consider looking into the relative contributions of genetic variation and epigenetic modification to the observed phenotypic variation in invasive plant species, and characterizing the three-way relationship between environmental cue, phenotypic plasticity and epigenetic changes. This framework also suggests that these studies should combine trait and molecular data and include comparative analysis of fitness functions between native and introduced ranges of a species (van Kleunen et al. 2018) and explore adaptive differentiation in invaders, while accounting for non-adaptive evolutionary changes. The unified research framework, therefore, may converge the parallel lines of research towards a better understanding of the mechanism of successful invasion.

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RESEARCH ARTICLE

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Native and introduced Argentine ant populations are characterised by distinct transcriptomic signatures associated with behaviour and immunity

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Abstract

Biological invasions can be influenced by trait variation in the invader, such as behavioural traits and ecological factors, such as variation in pathogen pressure. High-throughput nucleotide sequencing has increased our capacity to investigate the genomic basis of the functional changes associated with biological invasions. Here, we used RNA-sequencing in Argentina and California, Australia and New Zealand to investigate if native and introduced Argentine ant populations were characterised by distinct transcriptomic signatures. We focused our analysis on viral pressure and immunity, as well as genes associated with biogenic amines known to modulate key behaviour in social insects. Using a combination of differential expression analysis, gene co-expression network analysis and candidate gene approach, we show that native and introduced populations have distinct transcriptomic signatures. Genes associated with biogenic amines were overall up-regulated in the native range compared to introduced populations. Although we found no significant variation in overall viral loads amongst regions for viruses known to infect Argentine ants, viral diversity was lower in most of the introduced range which was interestingly associated with down-regulation of the RNAi immune pathway, primarily directed against viruses. Altogether, our data show that Argentine ant populations exhibit range-specific transcriptomic signatures, perhaps reflecting regional adaptations that may contribute to the ecological success of introduced populations.

Keywords

Argentine ant, Biogenic amines, Biological invasions, Immunity, RNA-seq, Viruses

Introduction

Exotic species are commonly transported around the world as inadvertent stowaways in cargo and can sometime become invasive and pose great threats to biodiversity, agriculture and other human activities (Hulme 2009). Through population bottlenecks or novel evolutionary forces, both during the introduction process and in the introduced range, introduced species can experience rapid changes and sometimes enhanced ecological success (Sakai et al. 2001; Suarez and Tsutsui 2008). Some traits and ecological factors, such as behavioural variation and pathogen pressure, are key determinants of invasion success and, therefore, quantifying variation in these may be particularly relevant for understanding biological invasions. High-throughput sequencing has increased our capacity to investigate the genomic basis of the functional changes associated with biological invasions (Rius et al. 2015). Here, we used RNA-sequencing data to investigate possible functional variations between native and introduced populations of the Argentine ant (*Linepithema humile*), a globally successful pest. We focused our analysis on genes associated with biogenic amines, which modulate key behaviour in social insects (Kamhi et al. 2017), as well as immunity and associated virus diversity.

Some behavioural traits are regarded as major drivers of biological invasions and have been specifically suggested in the context of ant invasions (Holway and Suarez 1999; Phillips and Suarez 2012; Silverman and Buczkowski 2016). For instance, low intraspecific aggression is thought to be a key driver of the ecological success of the Argentine ant in its introduced range (Holway et al. 1998). Other behavioural traits, such as increased foraging activity and high interspecific aggression, are also likely to influence the Argentine ant's invasiveness (Silverman and Buczkowski 2016). Neural pathways associated with hallmark behaviour of social insects, such as foraging and aggression, have been shown to be modulated by compounds, including the biogenic amines octopamine (OA), serotonin (5-HT) and dopamine (DA) (Kamhi et al. 2017). Interestingly, such behavioural traits are often correlated and can be considered as components of behavioural syndromes (Jandt et al. 2014). Biogenic amines can function as neurotransmitters (neuron-to-neuron communication), but can also function as neuromodulators and neurohormones, in which case they target larger regions of the brain or the body, respectively, and underlie specific behaviour (Hoyle 1985; Libersat and Pflueger 2004). In the context of biological invasions, variation in biogenic amine pathways could be associated with the regulation of behavioural syndromes that contribute to the success of introduced species. In social insects, expression of genes associated with biogenic amines, has been suggested to be associated with variation in social behaviour, including foraging and aggression (Liang et al. 2012, 2014; Kamhi et al. 2017; Friedman et al. 2018). Some of these genes have been shown to exhibit genetic variation associated with behavioural syndromes (e.g. in birds: Fidler et al. 2007;

Korsten et al. 2010) and can exhibit range-specific polymorphism (Mueller et al. 2013; Holtmann et al. 2016). Behavioural differences between the native and introduced Argentine ant populations might be restricted to specific behaviour in a specific context (Felden et al. 2018). To gain further insight into variation in the molecular basis of the Argentine ant's behaviour across its native and introduced ranges, we measured expression of genes related to the main biogenic amine pathways. We hypothesised that the molecular basis of behaviour, central to the fitness of ant societies, may exhibit range-specific expression profiles.

Pathogens often play an important role in biological invasions (Tompkins et al. 2011; Dunn et al. 2012; White and Perkins 2012). Social insects possess several characteristics that likely make them vulnerable to pathogens, including high densities of individuals within nests and social groups comprised of related individuals (Schmid-Hempel 1998). Introduced species, including invasive ants, can exhibit boom-and-bust population dynamics and pathogens may play a part in such dynamics (Simberloff and Gibbons 2004; Lester and Gruber 2016). The Argentine ant is known to harbour a number of microbes and invasion pathways have been shown to be associated with changes in microbial communities of invasive ants, including both losses and gains of pathogens and endosymbionts (Yang et al. 2010; Sébastien et al. 2015; Gruber et al. 2017; Lester et al. 2017; Viljakainen et al. 2018). Either loss or acquisition of pathogens along an introduction pathway could directly affect an invader's fitness and influence population dynamics and invasion success. Furthermore, under the Evolution of Increased Competitive Ability hypotheses (EICA), diverting resources from immunity or allocating resources to less costly immune pathways in response to relaxed pathogen pressure is thought to promote invasion success (Keane and Crawley 2002; Torchin et al. 2003; Lee and Klasing 2004). Homology-based analysis has revealed many immune genes that appear conserved across model organisms and Argentine ants (Smith et al. 2011). We measured variation in the expression of genes associated with the *JaK/STAT*, *RNAi*, *Toll* and *Imd* immune signalling pathways across the native and introduced ranges. To clarify the landscape of viral pressure in the native and invaded ranges, we also measured viral diversity and overall viral loads in our samples. We hypothesised that, if Argentine ant invasion were facilitated by variation in viral pressure, introduced populations may exhibit differential regulation of immune genes, thereby promoting invasiveness both directly and through beneficial resource re-allocation.

We examined possible functional adaptations underlying the success of a globally invasive pest, using RNA-sequencing across the native and introduced ranges of the Argentine ant. Are introduced populations characterised by differences in gene expression that could underpin behavioural variation? Are introduced populations characterised by a release from pathogens from the native range and does that translate into lower immune gene expression? To answer these questions, we investigated 1) variation in expression of genes associated with biogenic amines and 2) immunity, as well as 3) viral diversity in the native range (Argentina) and the introduced range in California, Europe, Australia and New Zealand.

Methods

Sampling and RNA library preparation

We used worker ants collected in Argentina, California, Australia and New Zealand from colonies maintained in standardised conditions for 20 days prior to sampling as described in Felden et al. (2018). Ants were collected at four different sites in each region, except in Europe that only included two distinct sites (Suppl. material 1: Table S1). Briefly, colonies were maintained in experimental colonies comprising 1,200 workers and four queens, fed daily with one mealworm (*Tenebrio molitor*, ≈ 150 mg) and 1 ml of 20% sucrose solution (or 1% sucrose for Low sugar colonies, i.e. samples $AR_{OTA\text{-}2}$, $CA_{RCH\text{-}2}$, $AU_{STA\text{-}1}$, $NZ_{HAS\text{-}3}$ and $NZ_{HAS\text{-}4}$). For the purpose of virus presence and load analysis only (i.e. not gene expression), we also included samples from colonies that were fed with one mealworm and octopamine in 1% sucrose solution (i.e. samples $AR_{OTA\text{-}3}$, $CA_{RCH\text{-}3}$, $AU_{STA\text{-}2}$, $AU_{STA\text{-}3}$; see Felden et al. 2018). Foraging workers were sampled and stored in ice-cold RNALater (Invitrogen, USA). Tubes of ants in RNALater were kept at 6 °C for 24 h, and at -20 °C in the country of origin until shipped to New Zealand where they were stored at -80 °C for up to six months until RNA was extracted.

Ant heads with antennae were separated from bodies in RNALater under a stereomicroscope and total RNA was extracted from 20 pooled heads and antennae of workers from the same colony. Samples were then briefly washed with ice-cold phosphate-buffered saline (PBS) to remove RNALater that can affect extraction quality. RNA was extracted using an in-column Trizol-based purification kit, using the manufacturer's recommended methods (Direct-Zol Microprep, Zymo Research, USA). RNA integrity was confirmed and quantified with an RNA 6000 Nano chip on the Agilent 2100 Bioanalyzer (Agilent Technologies Co. Ltd., Diegem, Belgium), according to the manufacturer's instructions. Extracted RNA was stored in RNAStable (Biomatrica Inc., San Diego, USA) and sent to BGI (Shenzen, China) for Illumina Hi-Seq sequencing. Overall, 30 head/antenna libraries were sequenced in the native and introduced ranges, including six replicates from Argentina (four sites), seven from California (four sites), three in Europe (two sites), seven from Australia (four sites) and seven from New Zealand (three sites) (see details in Suppl. material 1: Table S1). Four libraries that were experimentally treated with octopamine (OA) for another study were discarded from the gene expression analysis and only used in the virus analysis, as experimental treatment likely affected gene expression. Samples were sequenced as 150 base paired-end barcoded mRNA TruSeq libraries, aiming to generate 4 GB of data per sample. Pre-processing at BGI included the removal of reads with more than 10% values missing, reads with more than 10% of bases with quality scores $Q < 20$ and removal of adapters.

Read alignment and Argentine ant transcript quantification

Computationally demanding analyses were performed on Victoria University of Wellington Science Faculty's High Performance Computing Facility. Clean paired-end reads were aligned to the Argentine ant reference genome (Smith et al. 2011) with *HISAT* 2.1.0 using default parameters (Kim et al. 2015) to produce sample-specific BAM files. Overall, the average read alignment rate to the Argentine ant genome was 89.66%. *StringTie* 1.3.4 (Pertea et al. 2015) was used to generate GTF files from the BAM files, using the *-e* argument to restrict the assembly to transcripts matching the reference (Lhum_UMD_V04, accession number GCF_000217595.1). We generated a raw transcript counts matrix at the gene level using *StringTie*'s authors' *prepDE.py* script.

Differential gene expression analysis

To detect the most differentially expressed genes between the native and the introduced ranges, we followed part of the guidelines outlined in Law et al. (2016). Briefly, we imported the raw counts matrix into *R* as a *DGEList* object using the *edgeR* 3.22.3 package (Robinson et al. 2010), together with one of the GTF files created by *StringTie* as a source of information for each gene. To filter the raw counts matrix for low expressed transcripts, we first computed counts per million (CPM), discarded transcripts expressed in less than three samples at more than 1 CPM and then applied a TMM normalisation on the filtered raw count data. We then used the *limma* 3.36.5 (Ritchie et al. 2015) in *R* to perform a series of four pairwise differential gene expression analyses between Argentina and each region of the introduced range included in the dataset (i.e. California, Europe, Australia, New Zealand). We transformed TMM-normalised raw counts filtered for low expressed transcripts into log2-counts per million (log2CPM) with the *limma/voom* function and fitted a linear model for each gene on this post-filtering TMM-normalised log2CPM matrix. We selected the most differentially expressed genes within each pairwise comparison using a FDR cut-off of 0.05 and an absolute fold change > 1.1. To identify genes consistently differentially expressed between the native and the introduced range, we selected the overlap of the differentially expressed gene in all four pairwise comparisons, which yielded a set of 130 genes. In order to gain a broader functional assessment of the lists of differentially expressed genes between the native and introduced ranges, we undertook gene ontology (GO) enrichment analysis. First, we used BLASTx to determine the closest matches of all Argentine ant reference genes (assembly Lhum_UMD_V04, accession number GCF_000217595.1) against the Swissprot database (The Uniprot Consortium 2017; downloaded 28/02/2019). Then, we imported the BLASTx output as a XML file into BLAST2GO and proceeded to the mapping and annotation step in order to run Gene Set Enrichment Analyses (GSEA) restricted to Biological Functions. The list of differentially expressed genes was ranked based on fold-change.

Weighted Gene Co-expression Network Analysis (WGCNA)

WGCNA (in *WGCNA* 1.64-1, Langfelder and Horvath 2008) allows the detection of modules of co-expressed genes that can be correlated to factors such as phenotypic traits and is an alternative approach to detect genes of interest to that of a classical differential gene expression analysis as described above. To examine transcriptome-wide expression patterns associated with range (native or introduced), we analysed the post-filtering TMM-normalised log2CPM matrix produced prior to the differential gene expression analysis using WGCNA. We followed WGCNA authors' guidelines to further detect low expressed transcripts and outlying samples. We then used the scale-free topology criterion to select a soft thresholding power to build the gene co-expression network (Langfelder and Horvath 2008; Suppl. material 1: Figure S2). We used range as a numerical variable (0 for native populations and 1 for introduced populations) to investigate the correlation between the eigengene of each module and range. We investigated GO enrichment of the module that was the most correlated with range as outlined above in the DGE analysis section, except that genes were ranked based on module membership. Module membership indicates how the focal gene expression is correlated with the module eigengene expression profile (Langfelder and Horvath 2008). Highly connected genes that exhibit high module membership are likely to have particular biological relevance.

Candidate gene approach

To determine differences in gene expression associated with biogenic amines and immunity, we compiled a list of genes of interest, based on existing annotations of the Argentine ant genome (Lhum_UMD_V04 from Smith et al. 2011, listed in Suppl. material 1: Table S3, Table S4, respectively). First, we computed log-centred FPKM counts for genes of interest using the *rpkm* function in *edgeR* (Robinson et al. 2010), using the post-filtering TMM-normalised counts generated from the DGEList object described in the differential gene expression analysis section (i.e. prior to log2-CPM transformation). For genes belonging to immune signalling pathways, we computed the average expression per region (i.e. Argentina or one of the introduced regions) per gene and tested for differences in overall immune pathway expression. For immune pathways that contained 10 annotated genes or more, we analysed overall immune pathway gene expression, using data for all genes associated with each pathway of interest using linear models, with region as an independent variable. We tested for post-hoc pairwise differences amongst groups using the *multcomp/glht R* function (Hothorn et al. 2016). For immune pathways that comprised less than 10 annotated genes (i.e. *JaK/STAT* and *JNK*), we used a Kruskal-Wallis test, followed by post-hoc multiple comparisons if significant (Giraudoux et al. 2018). The genes of interest, associated with biogenic amines, were in limited number (i.e. 3 to 4 genes per biogenic amine pathway), hence we restricted the statistical analysis at the gene level and compared variation associated with range by pooling the data from the introduced regions.

Virome analysis

Reads that did not align to the Argentine ant genome were assembled *de novo* using *Trinity* 2.3.2 (Haas et al. 2013) using default parameters. We quantified transcript expression within *Trinity* 2.3.2 using *eXpress* (Roberts and Pachter 2012), yielding a TMM-normalised TPM matrix at the gene level. We then aligned the assembled transcripts to various reference databases to screen for and quantify viral transcripts. We used BLAST 2.2.3 (Camacho et al. 2009) to run BLASTx searches of the NCBI viral protein database (downloaded on 19/11/2018 from ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/), as well as of a more recently published Argentine ant novel viruses database (Viljakainen et al. 2018), using a *e-value* cut-off of 10^{-5}. We discarded transcripts that were less than 100 bases long and queries that returned < 90% identity with the target sequence. From the filtered BLAST outputs, we selected a single best hit per assembled transcript based on the highest bitscore. To compute viral loads, we first summed TPMs for all genes belonging to each identified virus (Suppl. material 1: File S1). Then, we used the Argentine ant library size, computed in the differential expression analysis section, to normalise viral loads to host tissue amongst samples.

Virus presence was verified via RT-PCR. Briefly, RNA was extracted from 10 whole ants per site (i.e. four extractions per region) following a similar Direct-Zol protocol as described above. Ants from Europe were sampled at only two different locations; hence these samples included two replicated extractions per site. A total of 250 ng of RNA from each extraction was pooled with respect to region so that each regional sample contained 1 µg of RNA. We prepared cDNA libraries using qScript cDNA SuperMix (Quanta Biosciences), using the manufacturer's instructions. Target-specific PCR conditions are given in Suppl. material 1: Table S6.

Results

Overall transcriptomic profile variation and most differentially expressed genes between the native and introduced ranges

Overall gene expression profiles clustered with region and range (Figure 1). We found highly variable numbers of differentially expressed genes between Argentina and introduced regions (Suppl. material 1: Figure S1, Table S2). For further analysis of range-specific transcriptomic signatures, we considered the 130 genes that were consistently differentially expressed between Argentina and all of the introduced populations (genes coloured in red in Suppl. material 1: Figure S1, File S1).

Genes that were consistently differentially expressed between the native and introduced ranges tended to be up-regulated in the introduced range (118/130 genes; Figure 2, Suppl. material 1: File S1). Gene set enrichment analysis indicated that this set of differentially expressed genes was only significantly enriched for

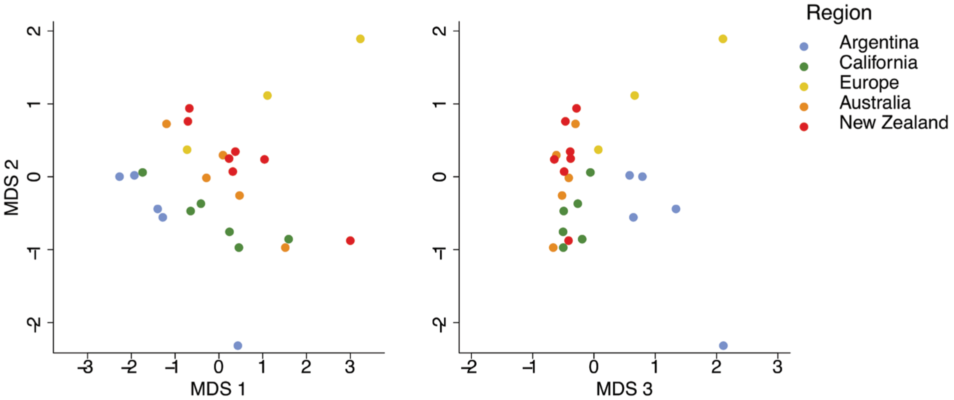

Figure 1. Gene expression clustering of samples of Argentine ants in their native and introduced ranges, based on multi-dimensional scaling of TMM-normalised counts per million (CPM) with low expressed transcripts filtered out. Euclidian distances between samples are computed from genes with the largest standard deviations. Regions are indicated as Argentina (AR), California (CA), Europe (EU), Australia (AU) and New Zealand (NZ) and collection site shown as subscript (details in Suppl. material 1: Table S1).

lipid transport (11 genes, *FDR* = 0.187, Suppl. material 1: File S2). We found significantly differentially expressed genes associated with immune pathways up-regulated in the introduced range, i.e. three proteins *Toll-like* (LOC105674719, LOC105668121 and LOC105674712), uncharacterised protein LOC105672003, recognition protein transcripts (three beta-1,3-glucan-binding protein-like (LOC105674418, LOC105674426 and LOC105674427), peptidoglycan-recognition protein-like LOC105676298 and MD-2-related lipid-recognition protein-like LOC105669917). We also found a number of genes associated with phototransduction that were differentially expressed between the native and introduced ranges, i.e. two transient receptor-like proteins (LOC105675038 and LOC105669468), guanine nucleotide-binding protein subunit beta-2 (LOC105678521), arrestin homologue (LOC105668394), rhodopsin and rhodopsin-like proteins (LOC105679645 and LOC105677743, respectively), phosrestin-2 (LOC105672477), blue- and UV-sensitive opsin transcripts (LOC105674733 and LOC105673714, respectively), protein aveugle (LOC105680182), retinol dehydrogenase 11-like (LOC105673325 and LOC105673325) and chaoptin (LOC105674278) (The Uniprot Consortium 2017).

Weighted Co-Expression Network Analysis

The gene co-expression network initially comprised 12 modules, which were reduced to 10 modules after merging based on expression similarity (Suppl. materi-

Figure 2. Details of the set of genes consistently differentially expressed between the Argentine ant native and introduced ranges, showing Z-score scaled log2-transformed TMMs with false discovery rate (FDR) < 0.05 and fold change (|FC|) > 1.1. Trees are based on average Pearson correlation of gene expression.

al 1: Figure S3). One of these modules was significantly (p < 0.001) and strongly correlated (r = 0.96) with range (Suppl. material 1: Figure S4). A total of 103 genes within the module were also present in the set of differentially expressed genes between the native and introduced ranges (Suppl. material 1: File S1). Gene set enrichment analysis of the module, associated with range, showed significant enrichment for redundant biological process terms related to immune signalling pathways (e.g. positive regulation of response to biotic stimulus, immune response-activating signal transduction, regulation of immune effector process with up to 36 genes per enriched term, *FDR* < 0.25, Suppl. material 1: File S3) and phototransduction (e.g. sensory perception of light stimulus, visual perception with up to 41 genes per enriched term, *FDR* < 0.25, Suppl. material 1: File S3). Thirteen genes overlapped between our list of candidate immune genes (Suppl. material 1: Table S4) and the co-expressed genes in the module, including five *Toll*-like transcripts (LOC105668121, LOC105668599, LOC105674013, LOC105674712 and LOC105678784), four beta-1,3-glucan-binding protein-like transcripts (LOC105673881, LOC105674418, LOC105674426 and LOC105674427), a *PGRP-LC* transcript (LOC105678063), *defensin-2* (LOC105975717), serine protease *nudel*-like (LOC105670067) and a *Toll/Imd*-associated uncharacterised protein transcript (LOC105672003) (The Uniprot Consortium 2017). Five of these transcripts (i.e. *Toll*-like protein LOC105668121, beta-1,3-glucan-binding protein-like transcripts LOC105674418, LOC105674426, LOC10567442, and uncharacterised LOC105672003) were amongst the 20% of most connected transcripts within the module (Suppl. material 1: Figure S5).

Candidate gene approach: genes associated with biogenic amines

Amongst the 14 genes selected for their association with biogenic amine pathways in Argentine ants, 10 serotoninergic, dopaminergic, octopaminergic and tyraminergic receptors exhibited expression levels that were significantly higher in the native range compared to the introduced range (Figure 3). A transcript coding for tyramine beta-hydroxylase (*TyrBH*) was also significantly up-regulated in Argentina and only the octopaminergic receptor *OctR1* and two serotoninergic receptors (*5-HTR-2A* and *5-HTR-2C*) expression levels were not significantly different between the two ranges.

Candidate gene approach: immune pathways

An immune pathway-specific analysis of gene expression revealed that genes associated with the RNAi pathway were consistently down-regulated in the introduced range compared to Argentina (p < 0.001 in all introduced regions; Figure 4a, Suppl. material 1: Table S5). The *JaK/STAT* followed a similar expression pattern, but was

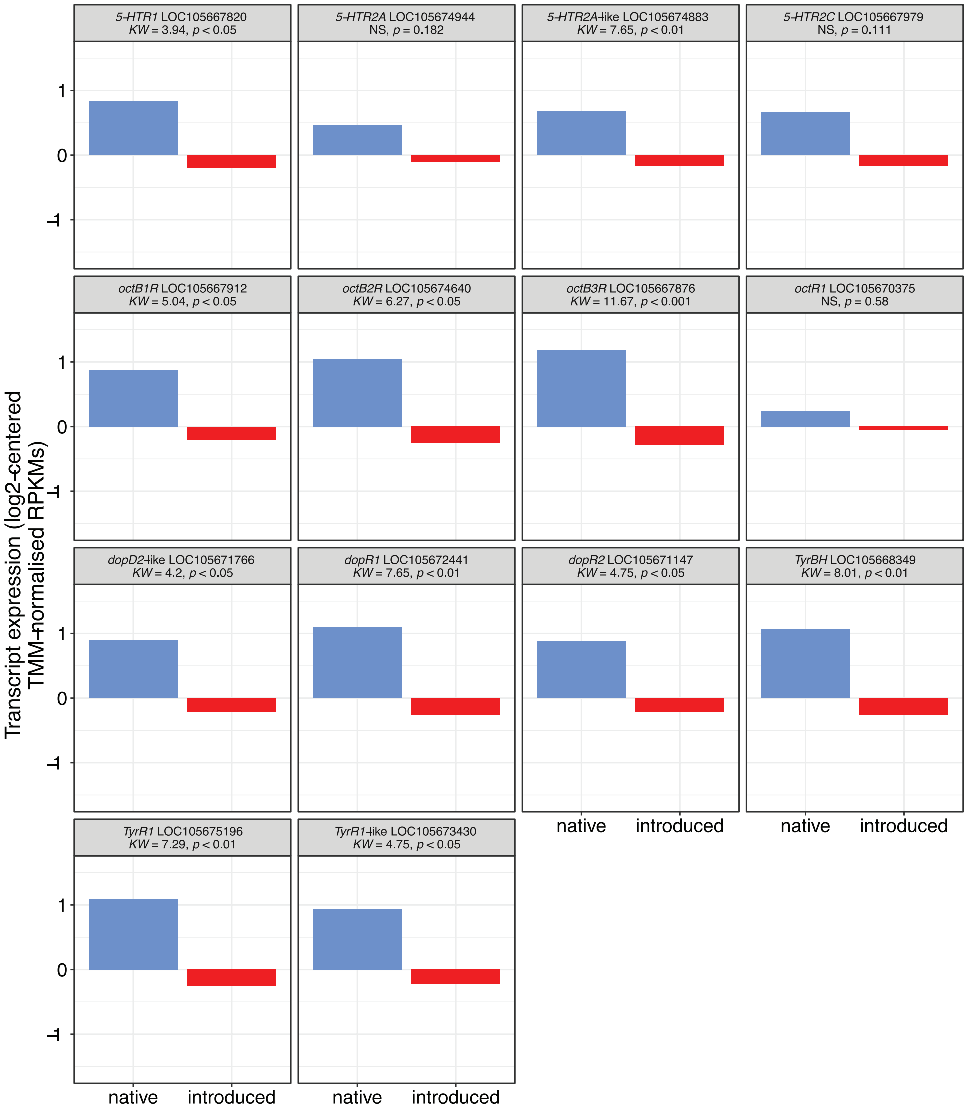

Figure 3. Log-centred TMM-normalised FPKM expression levels for genes associated with biogenic amine pathways. Results of Kruskal-Wallis tests are given above each plot facet. Full names and accession numbers of the genes included can be found in Suppl. material 1: Table S3.

only significantly down-regulated in Europe compared to Argentina ($p < 0.05$; Figure 4a, Suppl. material 1: Table S5). Conversely, genes associated with the *Toll* pathway were consistently significantly up-regulated in the introduced range (Figure 4e, Suppl. material 1: Table S5). Genes associated with *Imd* and *JNK* pathways did not exhibit a clear expression pattern between the native and invaded ranges (Figures 4c–d, Suppl. material 1: Table S5).

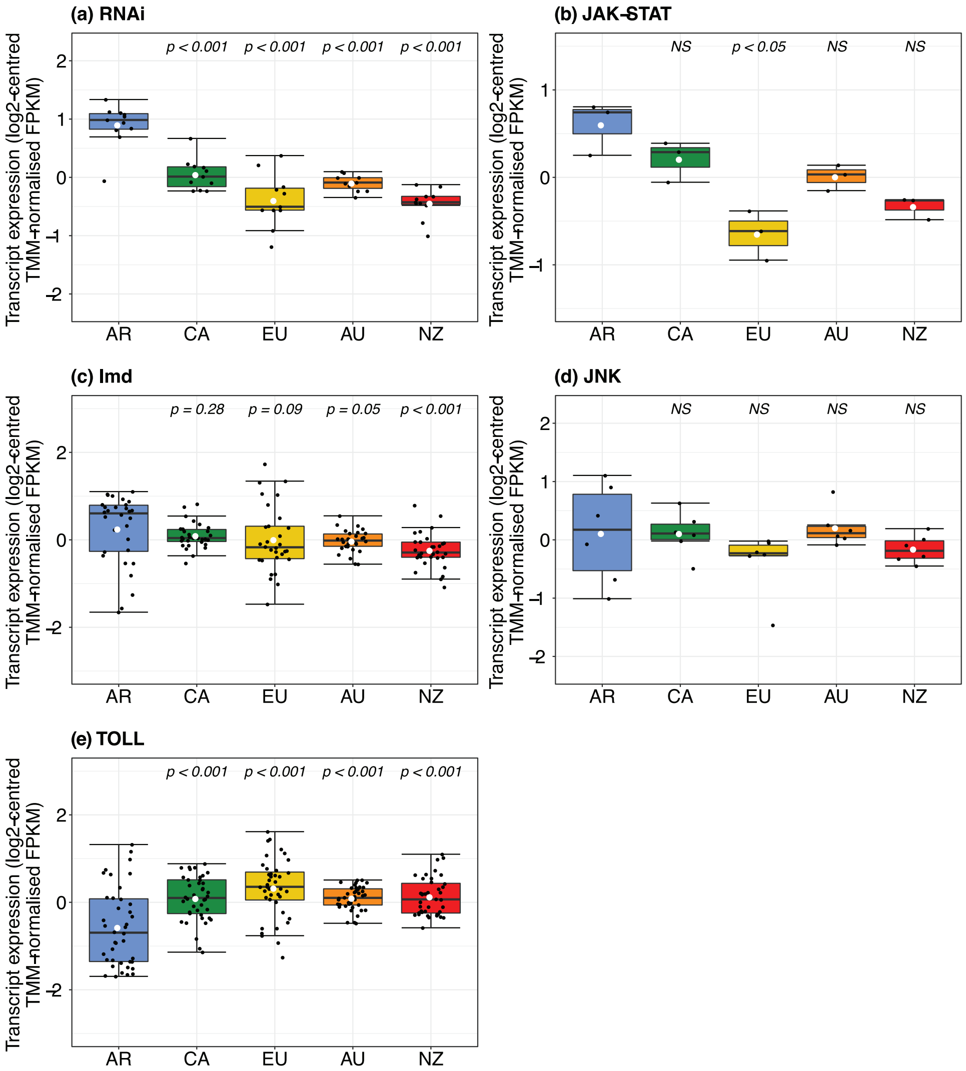

Figure 4. Variation in candidate immune gene expression in the Argentine ant native (Argentina, AR) and introduced (California, CA; Europe, EU; Australia, AU; New Zealand, NZ) ranges. Log-centred TMM-normalised FPKMs for genes associated with the immune pathways (a) RNAi, (b) JaK/STAT, (c) Imd, (d) JNK and (e) Toll. P-values for GLMs or Kruskal-Wallis tests, testing for differences between introduced regions and Argentina are given above each introduced range boxplot, compared to Argentina. Details of the genes, included in the datasets, are found in Suppl. material 1: Table S4 and full results of statistical tests in Suppl. material 1: Table S5.

Virome analysis

De novo-assembled transcripts that matched virus sequences using BLASTx searches were 102-2091 bases long (mean: 303.5 bases; median: 151; Suppl. material 1: File S4). Virus accumulation curves suggest a sufficient sampling size for most introduced regions, al-

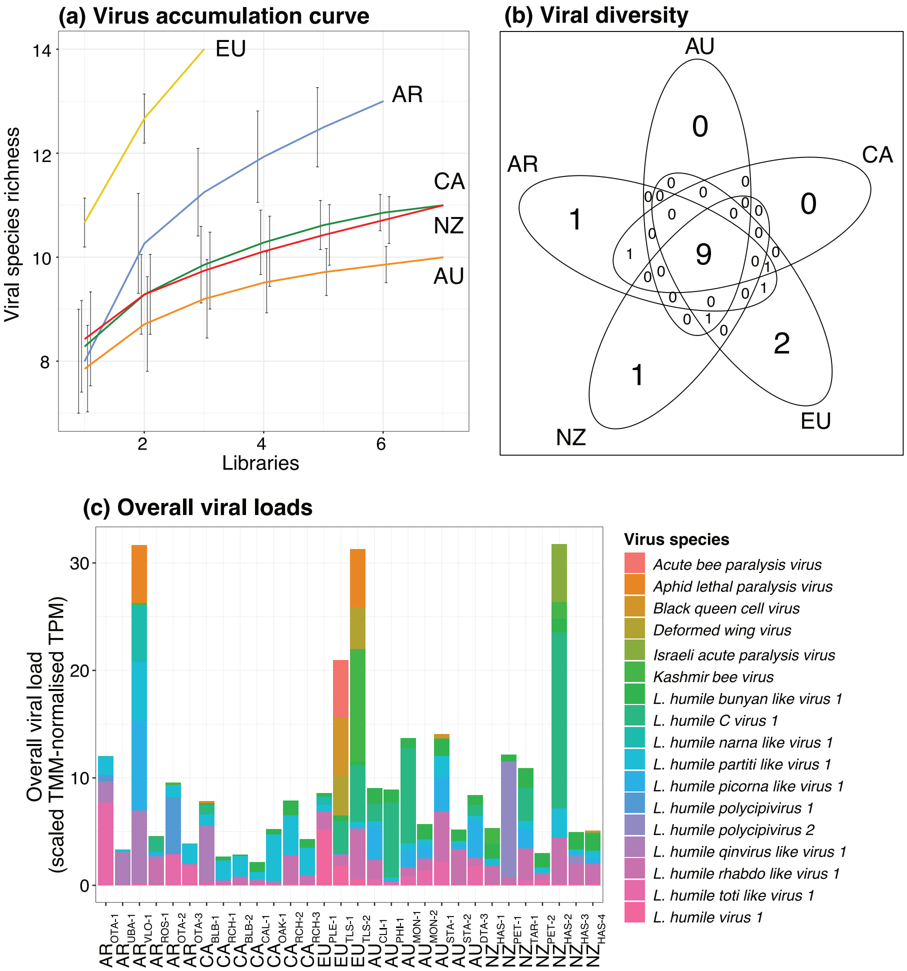

Figure 5. a Virus accumulation curves in Argentina (AR), California (CA), Europe (EU), Australia (AU) and New Zealand (NZ) as detected from RNA-seq data. Error bars indicate standard deviations **b** Venn diagram showing viral diversity and overlap amongst regions. Detail of the data is given in Table 1 **c** Overall viral loads per sample, showing contributions for all detected viruses in the dataset, expressed in TMM-normalised TPM scaled to the Argentine ant library size. Samples are identified with collection site and replicate in subscript (Suppl. material 1: Table S1).

though a plateau was not reached in Europe or the native range (Figure 5a). Fewer virus species were detected in California, Australia and New Zealand samples compared to Argentina and Europe and all study populations harboured a core virome of 9 known viruses (Figure 5b, Table 1). These ubiquitous viruses included *Linepithema humile picorna-like virus 1* (LhuPiLV1), *bunyan-like virus 1* (LhuBLV1), *partiti-like virus 1* (LhuPLV1), *toti-like virus 1* (LhuTLV1), *C virus 1* (LhuCV1), *rhabdo-like virus 1* (LhuRLV1), *polycipivirus 2* (LhuPcV2), *Linepithema humile virus 1* (LHUV-1) and *Kashmir bee virus* (KBV). Virus presence was confirmed with RT-PCR for a subset of viruses (Table 1).

Table 1. Viral diversity detected via RNA-seq, presence is indicated with 1, absence with a dash. Ubiquitous viruses are shown in bold. Virus presence confirmed by RT-PCR is shown with an asterisk.

	AR	CA	EU	AU	NZ
Lhu picorna-like virus 1	1	1	1	1	1
Lhu narnia-like virus 1	1	-	1	-	-
Lhu bunyan-like virus 1	1*	1*	1*	1*	1*
Lhu partiti-like virus 1	1*	1*	1*	1*	1*
Lhu qinvirus-like virus 1	1	1	-	-	-
Lhu toti-like virus 1	1*	1*	1*	1*	1*
Lhu C virus 1	1*	1*	1*	1*	1*
Lhu rhabdo-like virus 1	1*	1*	1*	1*	1*
Lhu polycipivirus 1	1*	-	-*	-	-
Lhupolycipivirus 2	1	1	1	1	1
LHUV-1	1*	1	1	1*	1*
Acute Bee Paralysis Virus	-	-	1	-	-
Black Queen Cell Virus	-	-	1	1	1
Aphid Lethal Paralysis Virus	1	1	1	-	-
Kashmir Bee Virus	1*	1	1*	1*	1*
Deformed Wing Virus	-	-	1	-	-
Israeli Acute Paralysis Virus	-	-	-	-	1
Total	13	11	14	10	11

Viral loads, expressed as the sum of viral transcripts detected, were extremely variable at the individual virus species level, as were overall viral loads computed by summing counts for all virus sequences (Figure 5c). We found no significant differences in overall viral loads amongst regions ($KW = 3.432$, $df = 4$, $p = 0.488$).

Discussion

We examined possible functional adaptations underlying the success of a globally invasive pest by investigating transcriptome-wide expression profiles associated with range in the Argentine ant. First, we identified the most differentially expressed genes amongst regions across the introduction pathway, as well as modules of co-expressed genes. We then further investigated gene expression profiles associated with immune pathways, as well as biogenic amine signalling. We also identified viral transcripts present in the libraries to measure viral diversity along the introduction pathway, as well as overall viral loads. Unsupervised multi-dimensional scaling analysis, based on normalised expression of all expressed transcripts, showed range and region-driven clustering. Similarly, hierarchical sample clustering, based on the most differentially expressed genes, showed perfect range-wise and close to perfect region-wise clustering. Functional analysis of differentially expressed genes in both analyses indicated a number of genes associated with a wide range of biological processes, including immunity. Further scrutiny at specific gene groups pointed to consistent range differences in genes associated with biogenic amines and key immune pathways. We also found lower viral diversity in the introduced range, highly variable viral loads between samples within and amongst regions, but no difference in overall viral loads amongst regions.

Behavioural traits key to the ecological success of ants, such as foraging and aggression, are modulated by biogenic amines (Kamhi and Traniello 2013; Kamhi et al. 2017). We investigated if expression of genes related to the dopaminergic, octopaminergic and serotoninergic neural pathways could be associated with the Argentine ant range, potentially contributing to its invasion success through, for instance, increased foraging activity, increased interspecific aggression and/or lower intraspecific aggression. Interestingly, we did not find behavioural differences in foraging activity between native and invaded ranges, and we found no variation in sensitivity to OA supplementation in the diet provided to the experimental colonies (Felden et al. 2018). Here, genes associated with biogenic amines, including OA, displayed clear range-specific expression pattern, suggesting an association with the introduction process. Biogenic amines are associated with a wide range of behaviour in insects, including key social behaviour in ants (Kamhi et al. 2017) and it is interesting that the vast majority of genes associated with biogenic amines exhibited the same trend. Although the insect brain is comparatively smaller than that of a vertebrate brain, it is a complex organ with largely heterogeneous regions that fulfil different functions and likely exhibit widely variable gene expression patterns. Further analysis of neuropil-specific expression patterns will be useful to elucidate variation in the molecular basis of behaviour.

In our study, all Argentine ant populations harboured a core virome of nine viruses, but we found lower viral diversity in most of the introduced range (i.e. California, Australia and New Zealand) compared to Argentina and Europe, where virus species richness was the highest. Variation in viral diversity has similarly been shown in another widespread invasive ant, the red imported fire ant, *Solenopsis invicta* (Yang et al. 2010). We found that the RNAi and *Imd* pathways, as well as the *JaK/STAT* and *JNK* pathways, are down-regulated in the introduced range. In honey bees, the *JaK/STAT* and RNAi immune pathways are primarily directed against viruses, whereas the *Toll*, *Imd* and *JNK* pathways are more ubiquitous and associated with defence against bacteria and other pathogens (Evans et al. 2006; Brutscher et al. 2015, 2017; McMenamin et al. 2018). Interestingly, genes associated with the *Toll* pathway in our results did not altogether show a clear range-specific expression pattern (i.e. candidate gene approach), but a number of *Toll*-associated genes were consistently up-regulated in the introduced range (differential gene expression analysis) and/or present in the gene module (WGCNA), suggesting a complex pattern of variation in immune responses between native and introduced populations. Changes in bacterial communities in the Argentine ant populations which we studied (Lester et al. 2017) with both acquisition and loss of bacteria may also be related to the variation in *Toll* genes expression that we observed. Host/pathogen interactions can be unique and much work remains to be done to fully understand specific immune responses associated with different pathogens and symbionts in Argentine ants. In Argentine ants, it has been shown that different viruses can trigger distinct immune responses, sometimes associated with specific virus families (Lester et al. 2019) and exposure to fewer viruses or a different viral community may result in population-wide differences in immune gene expression. Interactions amongst viruses within individual hosts and host communities appear to be complex (Viljakainen et al. 2018) and it is therefore hard to disentangle the respective weight of viral loads and diversity. Viral diversity may, however, be an informative metric in the context of biological invasions as it is less susceptible to variation at the population level.

Lower viral pressure in the introduced range may promote invasion success in two ways. First, ants are known to harbour a range of viruses (Valles et al. 2007, 2018; Sébastien et al. 2015; Cooling et al. 2017; Gruber et al. 2017; Viljakainen et al. 2018; Valles and Rivers 2019) and lower viral pressure may directly increase their fitness. For instance, viral infections in the red imported fire ant have been shown to influence colony foraging performance (Hsu et al. 2018). Second, in line with the EICA framework and supported by our results of lower expression of certain immune pathways primarily targeted against viruses, relaxed viral pressure may allow reallocation of resources away from immunity to other functions that increase the invader's fitness. Furthermore, different types of viruses are known to trigger different physiological responses (Lester et al. 2019). Therefore, plastic allocation of resources to specific immune responses with respect to variable viral exposure amongst regions may also increase the ant's competitive abilities and persistence of populations (Lester and Gruber 2016).

A significant proportion of genes up-regulated in the introduced range, compared to that of the native range, were surprisingly associated with vision. Some ant species heavily rely on vision to orientate themselves while foraging (Cheng et al. 2014), but Argentine ants have comparatively smaller eyes and are likely to have under-developed vision. Chemoreception appears to be the primary modality of Argentine ant's sensory biology, as indicated by the large number of chemoreceptor genes in their genome (Smith et al. 2011). Nevertheless, our results may indicate that vision has been overlooked in past studies. Alternatively and partly because gene annotation in non-model organisms is rarely definite, these genes may have been co-opted for other functions in Argentine ants. Throughout our analyses, we also found many genes associated with functions that are difficult to tie to specific biological processes and current resources do not allow for more than speculation on their association with the Argentine ant invasion. Our study does, however, point to several genes and gene families that are worthy of further investigation towards understanding patterns of invasiveness in Argentine ants and other species.

The introduced Argentine ant populations included in this study (i.e. California, Europe, Australia and New Zealand) all belong to the same global 'supercolony' characterised by high genetic similarity and absence of intraspecific aggression, which suggests a common origin (Corin et al. 2007; Van Wilgenburg et al. 2010; Vogel et al. 2010; Suhr et al. 2011). It is possible that the similarity in gene expression patterns, which we observed within the introduced range, is the result of a founder effect at the time of the primary introduction instead of a response to new selective forces associated with the introduced range, as hypothesised in Felden et al. (2018). In order to elucidate the underpinning causes for such variation in gene expression between native and introduced ranges, further investigations should include quantification of genetic variation in functional or regulatory loci related to the signalling pathways discussed herein in these populations, as well as populations from independent introduction events (e.g. Catalonian supercolony in Europe).

Conclusions

We found that native and introduced Argentine ant populations exhibit distinct transcriptomic signatures. Genes associated with biogenic amines were consistently upregulated in the native range, suggesting variation in the molecular basis of behaviour between the native and introduced range. We also observed lower viral diversity in most of the introduced range, which was associated with differential regulation of immune pathways, most notably in the RNAi pathway involved in defence against viruses. We provide the first evidence that native and introduced Argentine ant populations are characterised by transcriptomic variation that may reflect region-specific functional adaptations and contribute to the invasion success of the Argentine ant.

Data accessibility

Supplementary information, scripts and data can be downloaded from GitHub at https://doi.org/10.5281/zenodo.3351674; RNA-seq reads can be accessed on the NCBI SRA repository (BioProject ID PRJNA553098, http://www.ncbi.nlm.nih.gov/bioproject/553098)

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Supplementary material 1

Supplementary information

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Data type: molecular data

Explanation note: **File S1**: List of DEGs in the differential expression analysis and genes in module highly correlated with range in WGCNA (showing overlap between the outputs). **File S2**: Gene Set Enrichment Analysis for list of DEGs. **File S3**: Gene Set Enrichment Analysis for genes in module highly correlated with range. **File S4**: BLASTx output indicating best matches of de novo-assembled transcripts and published viral references. **Table S1**: Sampling sites for libraries included in the study. **Figure S1**: Volcano plots showing pairwise comparisons of genes differentially expressed between Argentina and each of the introduced regions. **Table S2**: Number of most differentially expressed genes between Argentina and introduced regions. **Figure S2**: Diagnostic plot for WGCNA parameter tuning. **Figure S3**: Module clustering and gene dendrogram. **Figure S4**: Heatmap showing eigengene-range correlation for all modules. **Figure S5**: Gene significance vs connectivity in module highly correlated with range. **Table S3**: List of candidate genes associated with biogenic amines. **Table S4**: List of candidate genes for immune pathways. **Table S5**: Output of the statistical tests for differences in immune genes expression between each introduced region and Argentina. **Table S6**: PCR conditions for confirmation of virus presence.

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