Pollinator selection against toxic nectar as a key facilitator of a plant invasion

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Abstract

Plant compounds associated with herbivore defence occur widely in floral nectar and can impact pollinator health. We showed previously that *Rhododendron ponticum* nectar contains grayanotoxin I (GTX I) at concentrations that are lethal or sublethal to honeybees and a solitary bee in the plant's non-native range in Ireland. Here we further examined this conflict and tested the hypotheses that nectar GTX I is subject to negative pollinator-mediated selection in the non-native range– but that phenotypic linkage between GTX I levels in nectar and leaves acts as a constraint on independent evolution. We found that nectar GTX I experienced negative directional selection in the non-native range, in contrast to the native Iberian range, and that the magnitude and frequency of pollinator limitation indicated that selection was pollinator mediated. Surprisingly, nectar GTX I levels were decoupled from those of leaves in the non-native range, which may have assisted post-invasion evolution of nectar without compromising the anti-herbivore function of GTX I (here demonstrated in bioassays with an ecologically relevant herbivore). Our study emphasizes the centrality of pollinator health as a concept linked to the invasion process, and how post-invasion evolution can be targeted towards minimising lethal or sub-lethal effects on pollinators.

Introduction

The occurrence of toxins in nectar may appear paradoxical since this is the primary reward for pollinators [1, 2], but it is none-the-less widespread across many plant families [3-5]. While the occurrence of toxic or deterrent phytochemicals in nectar could be maladaptive for plant fitness if they impact pollinator health, they may also increase exclusivity or fidelity of pollinator visitation and thereby the efficiency of pollen transfer [3, 6]. However, whether nectar phytochemicals are beneficial or detrimental to plant fitness can also be context dependent [7]. Yet despite both these potential beneficial and detrimental effects, pollinator-mediated selection either for or against so-called 'toxic nectar' has still yet to be empirically demonstrated in natural plant populations.

As a model system, we examined natural selection on toxic nectar in native and non-native populations of *Rhododendron ponticum* L. (Ericaceae). Species in this genus constitutively express diterpene grayanotoxins (GTXs) throughout most plant parts that are toxic to a wide range of insect and other animal species [8-11]. We previously reported that GTXs occur in *R. ponticum* nectar [12], and that while some pollinators such the buff-tailed bumblebee (*Bombus terrestris* L.) can consume GTX at naturally occurring nectar concentrations without adverse effects, other bee species, including honeybees and the solitary mining bee (*Andrena scotica*, Pérez), cannot [13]. Observed lethal and sub-lethal effects resulted from exposure to grayanotoxin I (GTX I) at ecologically relevant nectar concentrations, whereas the deacetyl derivative, grayanotoxin III (GTX III), was non-toxic. Our past work in this system also showed that GTX I, but not GTX III, was notably absent or significantly reduced in non-native populations in Ireland [12]. Given that pollen limitation may be common in non-native *R. ponticum* populations [14], this suggests that pollinator-mediated selection and loss of nectar toxins could have played a central role in facilitating invasion. Further analysis of this model system thus has the potential to reveal a better understanding of the benefits, trade-offs, and ecological significance of toxic nectar for plants, especially in terms of how plants evolve to optimise interactions with mutualists and antagonists. In this study, we consider the consequences of toxic nectar for pollinator health, and if and how invasive plants can evolve to maximise the services of pollinators while maintaining their defence against herbivores.

Beyond their potential influence on pollinator health, GTXs also serve as highly effective herbivore antifeedants in *Rhododendron* species [8-11]. Phenotypic correlation between defence-related compounds in nectar and other plant parts (such as leaves and phloem) appears to be a common phenomenon across plant families [5, 15-17]. Thus, the potential for conflicting pressure on GTXs from pollinators and herbivores exists across all plant parts. To investigate this possibility, we employed a path analysis framework to assess the direction and magnitude of phenotypic selection on leaf, flower,

and nectar GTXs in native and non-native *R. ponticum* populations. This approach allowed us to devise a realistic path model – reflecting a foliar biogenesis of grayanotoxins [18, 19] leading to linked expression in flowers and nectar – to quantify the extent to which phenotypic selection on a certain plant part was imposed directly, and indirectly (i.e., arising from phenotypic linkage with other plant parts). Complementary to phenotypic selection analysis, we also undertook manipulative experiments with pollinators and herbivores to examine their roles as potential selective agents.

The main objective of this study therefore was to examine if pollinators potentially drive post-invasion evolution of nectar and an important plant defence trait, and thereby act as key facilitators of invasion by the entomophilous species *Rhododenon ponticum*. In particular, we tested the hypotheses that: 1) the direction and magnitude of natural selection on nectar GTX I varies across the native and non-native range of *R. ponticum*, consistent with the reduced levels observed in non-native populations; 2) pollinators are important drivers of this selection in the non-native range (i.e., pollinator limitation of plant fitness is correlated with nectar GTX I levels); 3) GTX I levels in nectar are phenotypically correlated with those in leaves and flowers, which should therefore also show reduced levels in the non-native range; and 4) that any reduction in leaf GTX I represents a trade-off owing to its adaptive value against herbivory (determined in a feeding bioassay with an ecologically important insect herbivore).

Methods

Location, traits and abiotic variables measured

The study was conducted in nine native populations of *R. ponticum* subsp. *baeticum* in southern Spain and northern and southern Portugal, and four populations in the species' non-native range in Ireland (Table S1). Between six and ten plants were sampled per population, which typically numbered about 20-30 flowering individuals. A minimum distance of 20 m was kept between individuals so as to reduce the chance of sampling ramets. For each plant individual, nectar, leaf and corolla material was collected, and floral morphological and abiotic variables were quantified. Nectar was collected from between 8-15 unbagged flowers using microcapillary tubes, and was pooled until ca. 1 µl was obtained per plant. This volume was more than sufficient to obtain large quantifiable peaks for GTX I in LC-MS analysis (see below). So as to standardise the time point of collection across individuals, nectar was sampled from flowers in their beta-phase of phenology around the time of stigma receptivity [20]. From each flower that nectar was sampled, the corolla and nearest sub-tending leaf were also removed and immersed in silica gel in snap-seal bags in a composite sample for each plant. The appropriateness of this sampling technique was supported by the fact that grayanotoxins are known to function as constitutive defences in plants (as opposed to being specifically induced by damage) [9]

and are comparatively stable in dried tissues and in solution [21, 22]. Nonetheless, care was taken not to damage any plant tissue until after nectar samples were collected. In the lab, water was removed by freeze drying nectar and oven drying leaf and flower samples at <50◦C. Dried flower and leaf samples (30 mg) were ground to a homogenous powder and extracted (3 X 20 ml) in MeOH, from which a 200 µl aliquot was transferred to analysis vials. Dried nectar was re-suspended in 200 µl MeOH for analysis. Quantification of GTX I was carried out by LC-MS analysis as previously reported [12]. Final values of GTX I were expressed as a concentration of dry weight of tissue (µg/mg dw). Mean corolla width (measured as the widest horizontal distance between the tips of petal wings) and corolla tube width (measured as the internal diameter of the corolla tube at its base) were recorded with dial callipers from five flowers per plant [23]. We previously reported that several microhabitat factors (canopy cover, aspect, elevation and irradiance) explained a significant amount of variation in nectar toxin levels in *R. ponticum* [12]. Where appropriate, we utilised these variables to control for the confounding effect of environmental heterogeneity in models featuring toxin levels as an explanatory variable.

Relative fitness and pollinator limitation

Maternal fitness was measured as total seed set per plant. Calculation of seed set in tall, profusely flowering shrub or tree species can prove challenging, and hence a sub-sampling approach is often employed [24, 25]. To obtain estimates of total seed set in *R. ponticum* plants, we first calculated mean seed set from 8-40 capsules (depending on flower abundance per plant). Established regression equations from native and non-native populations [20, 26] were used to estimate viable seed number based on mature capsule length. Viable and non-viable seeds are easily discerned in this species due to miniscule size and weight of the latter. To then estimate the total number of flowers per plant, we counted the number of flower trusses (racemes consisting of a pseudo-whorl of usually 9-12 flowers) per individual and multiplied this by the mean flower number (inclusive of those at pre and post-anthesis stage) obtained from 15-20 trusses. Although not all flowers mature into fruiting capsules, these measures are none-the-less highly correlated in *R. ponticum* [20]. Finally, we multiplied total number of flowers per plant by the mean seed set per capsule to afford total seed set. Relative fitness was calculated by dividing individual seed set by the native or non-native range mean.

A cohort of five individuals per population was selected for application of a supplementary pollination treatment to measure pollinator limitation. Although *R. ponticum* is self-fertile, optimal seed set occurs under out-crossing [26], and in particular due to intrapopulation cross-pollination [20]. The supplemental treatment thus consisted of application of recently dehisced anthers from neighbouring plants (≥ 35 m distance away) to receptive stigmas of target flowers, ensuring deposition of the long

viscin pollen threads. The treatment was implemented at the start of the flowering period (late April in the native range; early June in the non-native range) when the activity of important pollinators (e.g. bumblebees, and solitary bees) was apparent [27]. Both treated flowers, and non-treated control flowers at the same phenological stage, were tagged and collected just preceding capsule dehiscence (mid-October in the native range; late January in the non-native range), with an overall retrieval rate of 88 % (due to wind damage, natural excision etc.). Pollinator limitation was therefore assumed in plants where supplementally treated flowers exhibited significantly higher seed set than open-pollinated control flowers, according to one-tailed Welch's *t*-tests. The resultant *t*-value of this test was taken as a continuous, quantitative measure of the magnitude of pollinator limitation per plant. While the ability to differentially allocate resources to out-crossed flowers has been noted in some species [28, 29], we did not believe this to be a confounding issue in our measure of pollinator limitation given the large gradients and spatially consistent patterns which were subsequently observed.

Field and experimental assessment of resistance to herbivory

All plants from which traits were measured were also surveyed for herbivore damage at the same time as when pollinator treatments were initiated in the native range (see above) and in early to mid-July in the non-native range. These time points hence permitted sufficient current-season herbivore damage to accumulate, in addition to previous years' damage evident on older leaves. Due to the typically large size of shrubs, we assessed herbivore damage in 1 m^3 areas at the edge of individuals from ground level upwards. The total number of young and old leaves within this area was counted, and the number of leaves exhibiting herbivore damage were recorded for each age class. If present, the area of damage on leaves was usually consistent (ca. 10-15 % area removed). A generalist species of broad-nosed weevil (Coleoptera: Curculionidae: Entiminae) known to feed on *R. ponticum* in the non-native range [30, 31], the Black vine weevil (*Otiorhynchus sulcatus* Fabricius), was selected for bioassays and reared from larval stage in a glasshouse on strawberry plants. Bioassays with black vine weevils (BVWs) were conducted using late instar adults in pre-oviposition period; a phase lasting 3-6 weeks during which time they consume the most plant foliage. Thirty adults were placed into individual arenas (20 X 10 X 6 cm) and randomly allocated to three treatments: 1) a control artificial diet; 2) an artificial diet with GTX I incorporated at natural leaf concentrations; and 3) an artificial diet in which ten times the natural concentrations of GTX I was incorporated. Artificial diets for BVWs were constructed following established techniques [32, 33], which consisted of cellulose acetate disks (0.45 µm pore size) treated with water-dissolved sucrose and β-sitosterol phagostimulants at concentrations known to solicit high feeding rates [34]. Sample sizes (the number of BVWs per treatment) were constrained by the limited quantity of GTX I we were able to isolate from several kg

of dried *R. ponticum* flowers, as per methods previously reported [35]. However, since there is typically low between-individual variation in BVWs due to obligate parthenogenesis [36], we considered these sample sizes adequate. Experiments were conducted for a total of 11 days (with a single change of cellulose disks at day 5.5) in conditions maintained at ca. 21 °C and 85 % relative humidity [37, 38]. The cumulative area eaten (mm^2) from disks was quantified per weevil from digital scans using ImageJ analysis software (National Institutes of Health, Bethesda, Maryland, USA). For both field and laboratory assessments, results are reported in terms of resistance (i.e. 1 minus % herbivore damage).

Data analysis

Comparison of GTX I across ranges – Geographic variation in nectar, leaf, and flower GTX I levels was analysed in separate linear mixed models (LMMs) fit by restricted maximum likelihood estimation using the R package nlme [39]. As three separate LMMs were conducted, we employed Benjamini-Hochberg adjustment of *p*-values to reduce the familywise error rate. Non-native plants are known to have originated from Spanish as opposed to Portuguese populations [40], and for this reason we restricted range comparisons to the former only. Nectar, leaf, and flower GTX I levels were square root transformed (to improve normality) and fit in LMMs as dependant variables against range (native and non-native) as a fixed effect and population as a nested random effect – with microhabitat variables included as covariates. For model validation, standardised residuals were examined for normality, homogeneity and independence, including spatial autocorrelation [41]. Non-equal variance of residuals between populations was accounted for in the leaf GTX I model by incorporation of a variance correlation structure (based on population identity), which significantly improved model AIC (Likelihood-ratio test; $L = 20.3$, $p=0.016$).

Natural selection on plant toxin levels – Before implementing selection analyses, we first: A.) controlled traits for potential confounding effects of environmental heterogeneity, as strong abiotic-mediated covariance between traits and fitness can bias estimates of selection gradients [42, 43]; and B.) affirmed the legitimacy of pooling population data [44] in order to assess selection at the range level. Details of these steps are provided (see Supplementary Methods). Subsequently, estimates of directional selection were obtained for each range through multiple regression of relative fitness on standardized traits [45] within a path analytical framework – following terminology of Scheiner et al. [44]. For path models, a hypothesized causal structure between leaf, nectar and flower GTX I levels and relative fitness was assessed. In addition, we tested for non-linear selection on traits, including quadratic (disruptive/stabilizing) and correlational selection [45]. However, as no significant non-linear selection was detected (data not shown), we focussed on directional selection only. We

employed mean-standardization of traits to allow output of mean-standardized selection gradients (β_μ) from analyses, as a measure of intensity of selection. These are deemed superior where comparisons of the strength of natural selection are desired, for instance between traits, or across geographic space [46] – with the added advantage of their interpretation as fitness elasticities [47, 48]; the resultant change in relative fitness from doubling trait values.

Path and mediation analyses were carried out using the R package 'lavaan' [49] for structural equation modelling. Data for both ranges were assessed for multivariate normality by Mardia's test in the R package 'MVN' [50]. As neither dataset met this requirement, we opted for robust maximum likelihood estimation of path coefficients as a non-parametric alternative. Path model goodness-of-fit is reported as the Satorra-Bentler adjusted Chi-squared (χ^2), which can provide better approximation of *p*-values under non-normality. Following the estimation of path coefficients, mediation analysis was employed to test the significance of three parameters in path models: 1) direct selection gradients (β_μ) (assessed along forward-connected paths from a trait to fitness, inclusive of any mediation through intermediate traits); 2) indirect selection (assessed as paths which lead forward to fitness first through a backwards step); and 3) total selection differentials (denoted *s*; the sum of direct and indirect selection) [44, 51]. Selection differentials estimated within a path model are also referred to as the 'predicted covariance', as values will usually differ from as typically measured (i.e. through simple trait-fitness correlations) in the absence of causal structure [44, 52]. As fitness measurements were not taken and/or could not be retrieved on all plants, missing values were casewise deleted. Final sample sizes for path and mediation analyses were $n = 68$ (i.e., $n = 38$ for the native range and $n = 30$ for the non-native range). These sample sizes are ca. 45-60 % of the median sample size reported for plants in a systematic review on selection [53], and are at least ten times the number of model explanatory variables, as per standard guidelines [54].

Biotic selection pressures on plant toxin levels – Differences in the frequency and intensity of pollinator limitation between ranges were assessed through Pearson's Chi-square Test for Independence and by t-test, respectively. Following this, multiple regression analyses were conducted for each range, to examine potential biotic and abiotic determinants of pollinator limitation. In addition to nectar toxins, a range of floral morphological (corolla and tube width) and microhabitat variables (canopy cover, aspect, elevation and irradiance) were considered for inclusion in models as potential co-determinants. Multicollinearity was monitored using variance inflation factors (VIFs). Final regression models contained explanatory variables significant after Benjamini-Hochberg adjustment of *p*-values. A Generalised Linear Model (GLM) with quasi-binomial errors (to account for overdispersed proportional data) was used to determine if there were differences in resistance to

BVW among GTX I treatments (control, normal, x10). Post-hoc Tukey pairwise comparisons were used to determine which treatments were significantly different from one another, using the R package multcomp [55]), and corrected for multiple comparisons by Benjamini-Hochberg adjustment. The frequency of herbivore damage on plants in the field was analysed according to the factors of leaf age and range of provenance (i.e. native or non-native) using Pearson's Chi-square Test for Independence. To investigate whether observed levels of plant resistance (i.e. 1 minus % herbivore damage) in the field could be explained by leaf GTX I and other microhabitat variables (as listed above) we fitted GLMs for each range with a quasi-binomial distribution (to account for over-dispersion). The overall significance of GLMs was assessed through comparison with a null model, and McFadden's pseudo-R^2 were generated to assess model fit.

Results

Natural selection on plant toxin levels

Directional selection on plant toxin levels was apparent in *R. ponticum* (Fig. 1; Table 1), with our *a priori* hypothesis of causal linkage between leaf, flower, and nectar GTX I and fitness deemed adequately representative of the observed data in path models for the native (χ^2 = 0.86, df = 1, p = 0.347) and non-native range (χ^2 = 2.21, df = 1, p = 0.137). However, the intensity and direction of phenotypic selection on traits was not consistent among regions; with strong positive total selection on leaf, flower, and nectar GTX I observed for plants in the native range, in contrast to significant negative total selection on nectar GTX I in the non-native range (Table 1). This discrepancy is indicative of divergent selection acting on nectar toxin levels, and is consistent with the pattern of phenotypic differentiation in nectar toxin levels found between ranges (Fig. 2). In contrast, leaf and flower GTX I which were selectively neutral in the non-native range did not differ in their phenotypic expression between ranges (Fig. 2). Linkage in toxin levels across leaves and nectar, and leaves and flowers, also appeared altered between ranges (Fig. 1), in which a breakdown in phenotypic correlation was indicated in the non-native range.

Decomposition of total selection on traits into direct and indirect components revealed that total selection on nectar and flower toxin levels in the native range is the result of large indirect selection acting through leaves (Table 1). While in the non-native range, only direct selection on nectar toxin levels was observed. Within ranges, no instances of conflicting selection on traits were observed (Table 1).

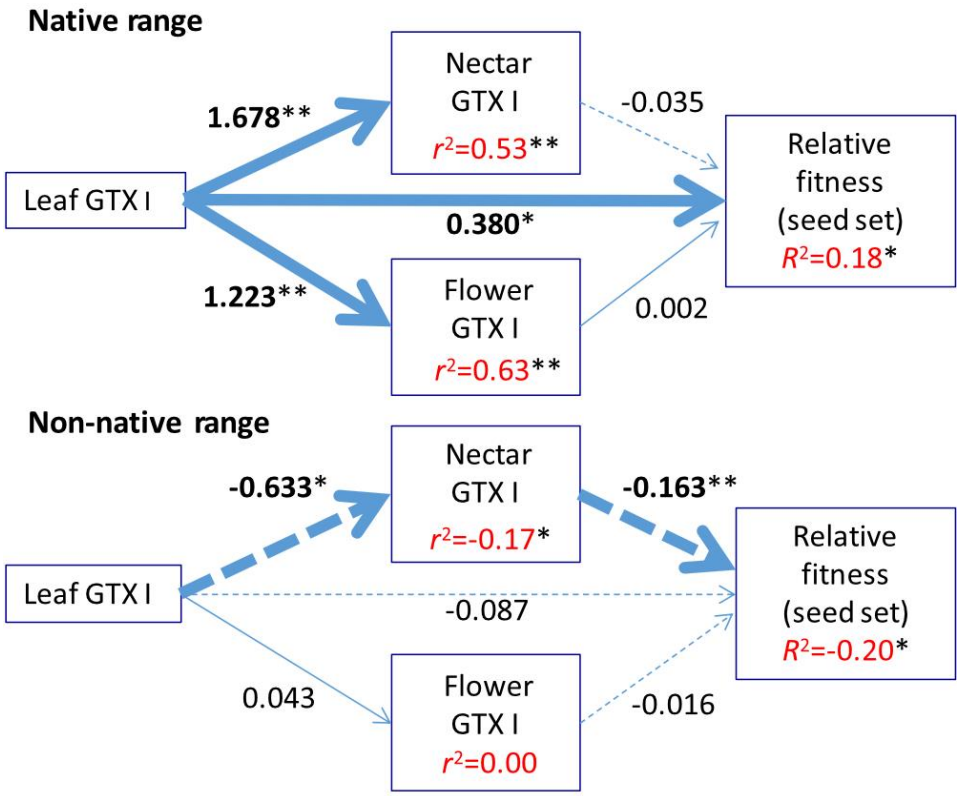

Figure 1. Solved path diagrams for directional selection on traits in the native (top) and non-native (bottom) range of *Rhododendron ponticum*. Mean-standardized path coefficients are presented, with dashed lines representing negative coefficients, and arrow width indicative of the strength of effect (bold values sig. at: * $P \leq 0.05$; ** $P \leq 0.001$). Direct selection is assessed along forward-connected paths to fitness, inclusive of any mediation through intermediate variables, and indirect selection as paths which lead forward to fitness first through a backwards step. The confounding influence of abiotic environment on traits was controlled for. Path analyses and multiple regressions are based on $N = 38$ and $N = 30$ for the native and invasive range respectively; and single correlations between tissues on $N = 53$ and $N = 30$.

Table 1. Mediation analysis of total selection on toxin levels in the native and invasive range, partitioned into direct and indirect components. Total selection (s) on a trait is the sum of all direct selection gradients (β) and indirect selection. Units are mean-standardized selection coefficients (± robust SE).

Range/Trait	Direct selection β (±SE)	Indirect selection (±SE)	Total selection† s (±SE)
Native			
Leaf	**0.324*** (±0.103)	n/a^	**0.324*** (±0.103)
Flower	0.002 (±0.090)	**0.393*** (±0.178)	**0.396*** (±0.140)
Nectar	-0.035 (±0.059)	0.642 (±0.339)	**0.607*** (±0.299)
Invasive			
Leaf	0.015 (±0.079)	n/a^	0.015 (±0.079)
Flower	-0.016 (±0.113)	0.001 (±0.004)	-0.015 (±0.113)
Nectar	**-0.163**** (±0.040)	0.055 (±0.052)	**-0.107*** (±0.049)

† Also referred to as 'predicted covariance' within context of a path model (see methods)

^ n/a due to the implied causal structure of path models

Bold values sig. at: * $P \leq 0.05$; ** $P \leq 0.001$

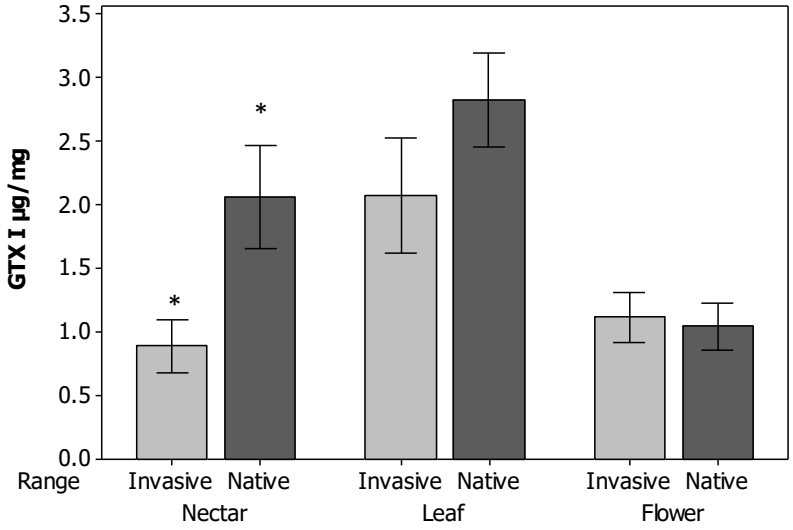

Figure 2. Mean toxin levels (GTX I µg/mg ± 95% CI) per dried sample type in the native and non-native range of *Rhododendron ponticum*. For nectar, leaf, and flowers, linear mixed models were fitted with 'range' as a fixed effect and 'population' as a nested random effect, and were controlled for abiotic environment. After adjustment for multiple comparisons, significant differences were detected between ranges for nectar (t= 3.82, $N_{[pops]}$= 13, $N_{[plants]}$= 87, p= 0.008), but not for leaves (t= 1.81, $N_{[pops]}$= 10, $N_{[plants]}$= 66, p= 0.162) or flowers (t= -0.07, $N_{[pops]}$= 10, $N_{[plants]}$= 66, p= 0.949).

Pollinators as drivers of selection on plant toxin levels

Plants in the native and non-native range differed significantly in the frequency of pollinator limitation experienced (χ^2 = 17.6, df = 1, p= ≤0.001); with seed set in 76 % of plants in the non-native range found to be pollen limited, compared to only 15 % of plants in the native range. There was also a difference in the intensity of pollinator limitation in plants between the native (mean = 0.95 ±0.34) and non-native range (mean = 4.24 ±0.57) (t-test: t= 5.22, df= 44, p= ≤0.001). Subsequently, we examined a range of biotic and abiotic factors to determine potential causes of pollination limitation in each range. The same pattern was observed within both ranges, in that plants which were more highly pollen limited possessed higher levels of nectar toxins and also wider flower corollas (Table 3). However, the strength of association between nectar GTX I and pollen limitation was more than three times greater in the non-native than the native range.

Table 3. Multiple regression analysis of determinants of pollination limitation. In addition to nectar toxins, a range of floral morphological and microhabitat variables (as listed in Methods) were considered for inclusion in models. Adjusted p-values are reported.

Range	**Coefficient** (± SE)	***t-value***	***p*-value**
Native[†]			
Nectar GTX I (µg/mg)	0.58 (±0.23)	2.52	0.020

Flower corolla width (mm)	0.22 (±0.07)	3.03	0.012
Non-native*			
Nectar GTX I (µg/mg)	1.83 (±0.76)	2.41	0.027
Flower corolla width (mm)	0.32 (±0.14)	2.24	0.039

† R^2 (adj) = 0.35 (F= 7.18, n= 26, p=0.004)

* R^2 (adj) = 0.27 (F= 4.43, n= 20, p=0.028)

Herbivores as drivers of selection on plant toxin levels

Evidence from controlled feeding experiments utilising an ecologically relevant herbivore of *R. ponticum* indicated that leaf GTX I functions as an important chemical defence conferring resistance to herbivory (Fig. 3a). Field observations in both the native and non-native range corroborated this finding, in that herbivory levels varied according to leaf age (χ^2 = 1159.2, df = 1, p= ≤0.001), with younger leaves exhibiting higher levels of damage and significantly less GTX I than older leaves (Paired t-test: t= 4.05, df= 36, p= ≤0.001). However, the apparent ecological value of leaf GTX I in conferring resistance was not consistent across ranges (Fig 3b), with herbivore damage to plants much more prevalent in the non-native rather than native range (χ^2 = 2181.8, df = 1, p= ≤0.001). Hence, in the non-native range, a significant association was observed between resistance and leaf GTX I levels (p= 0.008) together with canopy cover (p= 0.014) (quasi-binomial GLM: $F_{2,29}$= 5.2, p= 0.014, pseudo R^2= 0.30), while neither of these variables were significant in the native range (quasi-binomial GLM: $F_{2,37}$= 0.4, p= 0.700, pseudo R^2= 0.02).

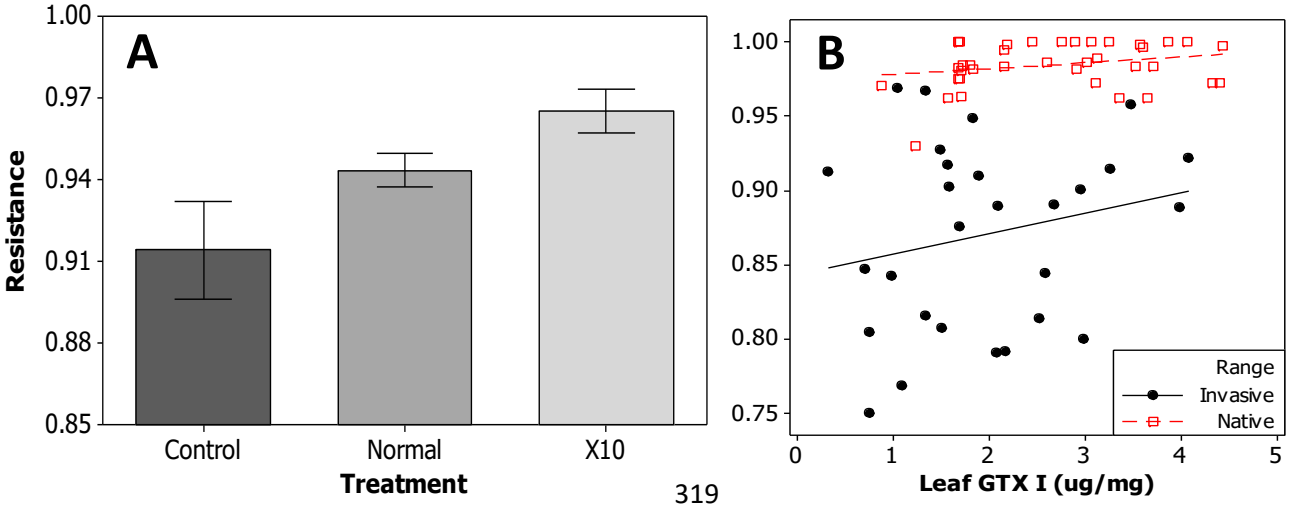

Figure 3. (A.) Resistance to Black vine weevil (*Otiorhynchus sulcatus*) feeding as conferred by grayanotoxin I (GTX I mean ± SE). Treatments represent artificial diets in which GTX I was absent (Control), or incorporated at average leaf levels in *Rhododendron pontcium* (Normal), or ten times this amount (X10). Each mean differed significantly from the other (at $p \leq 0.05$) according to one-tailed Tukey pairwise

contrasts (corrected for multiple comparisons); and (B.) the relationship between leaf GTX I levels and resistance of *R. ponticum* plants to herbivory in wild populations. A non-significant 'Range X Leaf GTX I' interaction revealed equivalency in this relationship across ranges (ANCOVA homogeneity of slopes: df= 61, p= >0.05).

Discussion

This study tested and confirmed the hypothesis that natural selection on a toxic plant chemical defence varied in direction and magnitude across the native and non-native range of an invasive species, given the expectation that mutualists and antagonists should exert conflicting selection pressures on leaves, flowers, and nectar. In the native range, positive total selection on toxin levels in flowers and nectar was as a result of an indirect selection on leaves; whereas in the non-native range nectar toxin levels experienced negative total selection, while other traits were selectively neutral.

Two lines of evidence supported the second hypotheses tested, that pollinators are important drivers of observed selection on toxic nectar. Firstly, the finding of negative selection on nectar GTX I in the non-native range, coupled with observed phenotypic change in GTX I that is specific to nectar (that is not seen for the comparatively less toxic GTX III [12]), provides evidence consistent with adaptive post-invasion evolution driven by pollinators. Furthermore, direct investigation of biotic selection pressures in both ranges revealed that plants that exhibited high nectar GTX I levels also experienced more pollen limitation. The vast majority of individuals in the non-native range were pollen-limited (compared to just 15 % in the native range). This high frequency and intensity of pollen limitation supports that nectar toxin levels were here subject to negative pollinator-mediated selection, given the importance of pollinators as selective-agents via seed production when plants are pollen limited [42, 59, 60]. Furthermore, due to the demonstrated sublethal post-ingestive effects of GTX I [13], established pollinators in the non-native range such as solitary bees may be differentially deterred by plant individuals on the basis of nectar toxicity. This type of preferential foraging behaviour by pollinators could be facilitated by the fact that high and low toxin producing plants tend to be spatially aggregated at the patch-level within plant populations [12].

Our findings led us to partially reject our third hypothesis, that GTX I levels in nectar are phenotypically correlated with those in leaves and flowers, which should therefore also show reduced levels in the non-native range. While such phenotypic correlation was indeed evident in the native range, this was not the case in the non-native range, where natural selection appears to have been able to act independently on nectar. While theory predicts that phenotypic expression of secondary compounds should become uncoupled across tissue types when these experience opposing selection pressures

[56, 57], such scenarios have seldom been tested or demonstrated [17]. Our study provides evidence of such an uncoupling, which has seemingly permitted non-native plants to reduce nectar GTX I levels without compromising the notable anti-herbivore function of GTX I in leaves and flowers. Far from a mere up-loading of phloem constitutions, the production of floral nectar in plants follows a complex, multi-stage process involving transport or *de novo* synthesis of components in various nectary ultrastructures [1, 58]. Such processes could hence form targets for the adaptive modification of nectar, which here may have permitted natural selection to act directly on nectar GTX I in the non-native range, while not compromising chemical defence in other tissues. In contrast, due to the observed linkage between tissue types in native plants, positive selection on leaf toxin levels resulted in large indirect selection on nectar toxin levels.

In relation to leaf GTX I levels and biotic selection pressures imposed by herbivores, we accepted our final hypothesis that that any reduction in leaf GTX I levels would represent a trade-off owing to its adaptive value against herbivory. Here, positive directional selection was observed on leaf and flower GTX I levels in the native range, consistent with the finding that GTX I conferred resistance against a generalist herbivore of this species, and that young leaves with lower toxin levels showed more herbivore damage throughout populations of both ranges. However, while there was a similarity between ranges in the general form of the relationship between leaf GTX I levels and herbivore resistance, this relationship was only significant in the non-native range, where *R. ponticum* has evidentially experienced a notable gain in levels of herbivore damage. This scenario represents a seemingly rare contradiction [61, 62] of the enemy-release hypothesis, which is often invoked to explain the success of invasive species in their non-native range. Hence, explanation as to finding that leaf and flower toxin levels were under positive selection in the native range, but not so in the invasive range, may therefore relate to the existence of other unmeasured relevant sources of herbivory.

Conclusions

Where interactions involving mutualists and antagonists are mediated by the same trait in plants, rarely are pollinators implicated as predominate selective agents [63]. This study therefore represents the first evidence of pollinator-mediated selection acting on a defence-related compound in nectar. These results also indicate how the possible microevolutionary adaptation of nectar by plants – which is generally held as the most important mediator of interactions with mutualists [1] – may facilitate colonization of exotic habitat, such as occurs in the invasion process. We conclude that pollinator-mediated selection and the subsequent loss of nectar toxins are likely to have played a key role in facilitating invasion by *R. ponticum*. These findings in addition emphasize the centrality of pollinator health as a concept linked to the invasion process, and how post-invasion evolutionary pressures can

minimise lethal or sub-lethal effects on pollinators. However, beyond only plant invasions, the generality of these findings on pollinator-mediated selection may in fact be broad – given the relatively widespread occurrence of toxic nectar amongst plant families [3, 5], and where species are distributed over large ranges throughout which conflicting selective pressures by pollinators and herbivores may occur.

Authors' contributions

All authors contributed to the design of the study. J.S. secured funding for the project, P.E. and J.S. conceived and designed the experiments, P.E. conducted the fieldwork and bioassay, and P.E. and P.S. conducted the chemical analysis. P.E. undertook the statistical analysis and drafted the initial manuscript. All authors contributed to the final article.

Competing interests

The Authors declare no competing interests.

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