Plant toxin levels in nectar vary spatially across native and introduced populations

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Summary

1. Secondary compounds in nectar can function as toxic chemical defences against floral antagonists, but may also mediate plant-pollinator interactions. Despite their ecological importance, few studies have investigated patterns of spatial variation in toxic nectar compounds in plant species, and none outside their native range.
2. Grayanotoxin I (GTX I) occurs in nectar of invasive *Rhododendron ponticum* where it is toxic to honeybees and some solitary bee species. We examined (i) geographic variation in the composition of nectar GTX I, as well as GTX III (which is not toxic to these species), in the native and introduced range of *R. ponticum*, (ii) how their expression is structured at patch and

landscape scales within ranges, and (iii) if climatic and environmental factors underpin spatial patterns.

3. While both GTXs varied within ranges, variation in GTX I, but not GTX III, was detected between ranges. GTX I expression was thus markedly lower or (in 18% of cases) absent from nectar in introduced plants. Spatial autocorrelation was apparent at both patch and landscape scales, and in part related to heat load interception by plants (a function of latitude, aspect and slope).
4. As expression of nectar GTXs was generally robust to environmental variation, and aggregated in space, this trait has the potential to be spatially discriminated by consumers. Given the specificity of change to GTX I, and its differential toxicity to some bee species, we conclude that its expression was likely to have been influenced during invasion by interaction with herbivores/consumers, either via pollinator-mediated selection or enemy-release from floral antagonists.
5. *Synthesis.* As the first demonstration of large-scale geographic variation and spatial structure in toxic nectar compounds, this work deepens our understanding of the chemical ecology of floral interactions in native and introduced species. Spatially explicit studies of nectar secondary compounds are thus required to show how the extent and structure of spatial variation may affect floral ecology. Future development of invasion theory should incorporate a holistic view of plant defence, beyond antagonistic interactions, which integrates the consequences of chemically defended mutualist rewards.

Key-words: chemical defence, grayanotoxins, invasion ecology, nectar chemistry, plant-herbivore interactions, pollination, spatial variation

Introduction

Toxic or deterrent secondary metabolites are produced by plants as a form of defence against herbivory and are virtually ubiquitous in tissues exploited by phytophagous insects (McKey, 1979, Agrawal and Weber, 2015). Although floral nectar primarily attracts and rewards pollinating animals, it may contain secondary compounds that have toxic or deterrent properties (Adler, 2000, Rhoades and Bergdahl, 1981, Baker, 1977). Surprisingly, so-called 'toxic' nectar is likely to be relatively widespread in plants. While this phenomenon was initially known to occur in ca. 20 phylogenetically diverse families (reviewed in Adler, 2000), identification of new sources has become increasingly frequent (Irwin et al., 2014). The types of toxins found in nectar are diverse, and include iridoid and cardiac glycosides (Lohaus and Schwerdtfeger, 2014, Manson et al., 2012, Richardson et al., 2015, Stephenson, 1982), toxic or otherwise deterrent phenolics (Baker, 1977, London-Shafir et al., 2003), a variety of alkaloids of indole, pyrrolizidine, tropane, purine and quinolizidine class (Wright et al., 2013, Boros et al., 2010, Kretschmar and Baumann, 1999, Masters, 1991, Adler et al., 2006), and structural combinations such as diterpene alkaloids (Gosselin et al., 2013, Manson et al., 2013).

When toxic or deterrent compounds are found in nectar, they tend to appear at considerably lower concentrations than in other plant parts (see Parachnowitsch and Manson (2015) and references therein). Their presence may indicate a defensive function against invertebrate and microbial antagonists such as floral larcenists and nectar-dwelling pathogens (Aizenberg-Gershtein et al., 2015, McArt et al., 2014, Gonzalez-Teuber and Heil, 2009). Floral larcenists include nectar robbers, which damage floral tissues in their attempts to access nectar, and small-bodied nectar thieves such as ants, thrips and pollen beetles – both robbers and thieves can consume large nectar volumes while rarely if ever providing pollination service (Irwin et al., 2010). The presence of defence compounds in nectar may therefore pre-empt such exploitation, or limit its impact to more benign levels (Stephenson, 1981,

Kaczorowski et al., 2014). In addition, toxic or unpalatable nectar compounds may serve as a filter against ineffective pollinators or pollinating guilds. For instance, evidence suggests that generalist butterflies, which are mostly poor vectors of pollen, are deterred by pyrrolizidine alkaloids likely to be found in the nectar of several plant families (Masters, 1991). However, deterring otherwise legitimate pollinators can also have fitness consequences for plants (Adler and Irwin, 2005, Adler and Irwin, 2012, Strauss et al., 1999), particularly where competition for pollinators is high (Gegear et al., 2007). While functioning as a filter against ineffective pollinators, toxic nectar can in this context preserve nectar for legitimate pollinators, provided that they are not intoxicated or repelled by these compounds (Liu et al., 2007, Gosselin et al., 2013, Nicolson et al., 2015, Masters, 1991, Thomson et al., 2015). In certain instances, consumption of nectar toxins may in fact confer physiological benefits to pollinators. Experimental studies have shown that these benefits may be direct, such as through improving memory of reward (Wright et al., 2013), or indirect, such as through reduction of intestinal parasite loads (Baracchi et al., 2015, Manson et al., 2010, Richardson et al., 2015). In contrast to the above adaptive hypotheses, however, the expression of secondary compounds in nectar could also (at least in part) be due to phenotypic linkage with other plant parts (Adler et al., 2006).

Although attention has focussed on the ecological function of toxic and deterrent compounds in nectar, surprisingly few studies have examined spatial variation in the quantitative and qualitative composition of these compounds. It is well-established that spatial variation in plant nectar traits influences the quality and distribution of floral resources encountered by flower visitors (Gijbels et al., 2014, Schmid et al., 2015, Leiss and Klinkhamer, 2005, Leiss et al., 2009), which can greatly impact pollinator foraging behaviour and fitness, and, in turn, the reproductive success of plants. In contrast, little information is available on phenotypic variation in nectar toxins, and knowledge of what constitutes typical intraspecific variation in species remains poor. In the only previous investigation of this kind to-date, large variation in nectar nicotine levels was noted within populations of *Nicotiana attenuata* Torr. ex

S.Watson (Kessler et al. 2012). In this study, plants which produced high nicotine levels repelled important hummingbird pollinators. This highlights the potential ecological ramifications of phenotypic variation in toxic nectar-producing plants. As with many foliar defence compounds (Moore and DeGabriel, 2012, Laitinen et al., 2005), a combination of genetic, developmental and environmental factors could underpin quantitative and qualitative variation of toxins in nectar at a range of spatial scales. For example, experimental studies with *Nicotiana* L. species indicated that nectar alkaloid levels varied according to nutrient availability, and can be rapidly induced in response to nectar robbing and foliar herbivory (Adler et al., 2006, Kaczorowski et al., 2014). Such processes could drive complex spatial heterogeneity in natural populations, and highlights the importance of better understanding the patterns, causes and consequences of variation in nectar toxins in plants.

In this study we investigated spatial variation in toxic nectar in a shrubby species, *Rhododendron ponticum* (L.). The species is native in the Black Sea region and as a Tertiary relict in the Iberian Peninsula, in disjunct regions of northern and southern Portugal and southern Spain. It is also naturalised and highly invasive in NW Europe, following deliberate introductions in the late 1800s (Dehnen-Schmutz and Williamson, 2006). Introduced populations descend from Spanish ancestors (i.e. *R. ponticum* subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti), and some introgression has occurred between *R. ponticum* and congenerics in parts of Britain (Milne and Abbott 2000), but most likely not in Ireland (Erfmeier et al., 2011, Stout et al., 2015). As in other Ericaceous genera, rhododendrons produce toxic diterpene secondary metabolites known as grayanotoxins (GTXs – reviewed in Qiang *et al.* (2011)), which are characterised by a polyhydroxylated tetracyclic ring structure. GTXs has a neurotoxic mode of action blocking sodium channel receptors and preventing their inactivation (Takeda and Narahashi, 1988). As sodium channels are nearly ubiquitous in animals, these compounds are toxic to a broad range of vertebrate and invertebrate organisms (Klocke et al., 1991, Oliver et al., 2015, Puschner et al., 2001, Tiedeken et al., 2016). Indeed the human toxicity of honey produced from the flowers of *R. ponticum*

(known as ‘mad honey’) has been known since antiquity (Koca and Koca, 2007). The two major grayanotoxins reported from *R. ponticum* are grayanotoxin I and its diacetyl derivate grayanotoxin III (Hough et al., 2010). Although there has been much focus on the mode of toxicity of GTXs produced by *Rhododendron* and other ericaceous genera, studies examining these compounds in an ecological context are few.

Mature plants of *R. ponticum* produce a profuse flowering display consisting of hundreds or thousands of large purple-pink zygomorphic flowers, with an average standing crop of 1.0 ± 0.7 µl nectar per flower (PA Egan, personal observation; *n* = 121 flowers). They are most frequently visited by social and solitary bee species in the native and introduced range and are particularly attractive to bumblebees including the buff-tailed bumblebee, *Bombus terrestris* L. (Mejias et al., 2002, Stout, 2007b, Stout et al., 2006). Overall rates of flower visitation are comparable between ranges, although the relative frequency of visits by social and solitary bee groups differs, with more social bee visits in the introduced range (Stout 2006). While *B. terrestris* appears unaffected by concentrations of nectar GTXs most typically encountered in the introduced range (Tiedeken et al., 2014), other pollinating insects such as honeybees and solitary species are here poisoned by GTX I (Tiedeken et al., 2016), and honeybees certainly avoid the plant (Stout et al., 2006). Since *R. ponticum* is strongly reliant on animal-pollinated seed production in the introduced range (Stout 2007b), deterrence of these medium-sized floral visitors (which may otherwise be effective pollen vectors) suggests a maladaptive consequence of nectar GTXs.

The defensive chemistry of mutualist rewards in introduced species is a rarely examined topic (Whitehead and Bowers, 2013). The present study thus represents the first spatial assessment of a nectar secondary compound in introduced species, and one of few biogeographical comparisons of nectar between native and introduced populations (Ollerton et al., 2012, Stout et al., 2006). Invasion theory makes several predictions in relation to the chemical defence of plant root and vegetative

structures in introduced species (Callaway and Ridenour, 2004, Cappuccino and Arnason, 2006, Doorduin and Vrieling, 2011, Joshi and Vrieling, 2005). In the case of chemically defended nectar and pollen, both enemy-release from floral larcenists and the need to facilitate a non-coevolved pollinator fauna could lead to altered defence allocation in introduced species. Thus the objectives of this study were: (i) to examine geographic variation in toxic nectar, in quantitative and qualitative terms, within and between the native and introduced range; (ii) to assess how this variation is spatially structured at patch and landscape scales; and (iii) to examine potential environmentally-induced variation in nectar GTX levels in response to climatic and environmental factors.

Material and methods

Field locations and sampling

Nectar of *R. ponticum* was sampled from 7-10 plants in nine populations covering the full extent of the native range in Spain and Portugal, and from seven populations throughout the introduced range in Ireland (Table S1 in Supporting Information). A minimum distance of 20 m was maintained between sampled plants in order to avoid sampling clonal ramets. Plant individuals were included regardless of the extent of herbivore damage, which was frequent (although rarely severe) in the introduced range, but less so in the native range (PA Egan, personal observation). For each sampled plant, 13 environmental variables were quantified, either in the field or derived from bioclimatic datasets. These included variables describing the biotic environment (canopy cover, habitat type), topography (elevation, slope, aspect) and climate (heat load index, irradiance, annual mean, minimum and maximum temperature and precipitation). The sampling resolution of these variables thus ranged from the plant level (ca. 30 m), to landscape scale (1 km^2). Further details of each, including method of quantification and/or original data source, are provided in Table S2.

In addition, one population was selected (pop. I2; Table S1) to examine how potential fluctuation in environmental conditions between years influences nectar GTX levels. This population was visited during the flowering period in early May in two sequential years. Collection of nectar from all sites was made in the field using 1 µl glass microcapillary tubes (Hirschmann, Germany), which were inserted into the nectar tube at the back of the flower. Flowers were randomly selected in order to avoid positional bias. An average of 1 µl was extracted per flower, and pooled from 6-14 flowers to obtain a minimum of 10 µl of nectar per plant. Care was taken to avoid damage to the corolla and nectaries in this process, so as not to contaminate samples. Samples were then frozen several hours after collection until the time of analysis.

Chemical analysis of nectar

Nectar samples were freeze-dried in vial inserts, and water content recorded by weight. Dried nectar was then re-suspended in 200 µl MeOH for analysis. LC-MS analysis was carried out on a Waters Alliance LC and ZQ MS detector, with 10 µl injection volume onto a Phenomenex Luna C18(2) column (150 X 4.0 mm i.d., 5 µm particle size) held at 30˚C. A gradient elution was employed consisting of a mobile phase of (A) MeOH, (B) H_2O, and (C) 1% HCO_2H in MeCN at a flow rate of 0.5 ml min^{-1} (A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min). For use as an analytical standard, several mg of GTX I were isolated from flowers of *R. ponticum* through process of solvent partitioning and prep-HPLC, adapted from previously reported methods (Chen et al., 2004, Elnaggar et al., 1980). A high (> 95%) purity was determined by ^{1}H and ^{13}C NMR and LC-MS, as described in Tiedeken et al. (2014). While trace levels of other GTXs may exist in this species, we restricted our analysis to GTX I and GTX III, as these were by far the most dominant GTXs in nectar (data not shown). Concentrations of these compounds in nectar were calculated based on constructed calibration curves. For this, peak areas of GTX I and a commercial standard of GTX III (purchased from Sigma Aldrich; ≥ 90% purity) were obtained from extracted ion chromatograms of the *m/z* 411.1 (GTX I; Rt = 8.1 min) and *m/z*

415.3 (GTX III; Rt = 6.71 min) pseudomolecular ions in negative electrospray ionisation mode. Final values of GTX were expressed as concentration in dry weight of nectar (μg/mg DW), to control for any environmental influences such as dilution or evaporation.

Data analyses

All analyses were conducted in R version 3.2.1 (R Core Team 2015). As both GTX I and GTX III data were Poisson distributed, values were square root transformed before use in all analyses. Results are reported in transformed units, unless otherwise stated.

Geographic and environmental variation

As GTX I and GTX III were quantified from nectar of the same plant individuals, and are biosynthetically related compounds (GTX III being the diacetyl derivative of GTX I), we initially conducted a multivariate analysis of variance (MANOVA) to assess the validity of modelling each GTX independently. The results of the MANOVA indicated that range explained a significant proportion of the variability in GTX I and GTX III (Wilks' $\lambda = 0.67$, $F_{2,97} = 23.5$, $P < 0.001$). Subsequent to a significant MANOVA result, we proceeded to examine geographic and environmental variation in nectar GTX I and GTX III levels (and nectar water content) independently in linear mixed models (LMMs). Only Spanish populations of the native range were considered in LMM analyses, as these formed the original source from which introduced populations are derived (Milne and Abbott, 2000). First a saturated model was fitted for each response variable (i.e. GTX I, GTX III and nectar water content), in which the factor of range and environmental covariates (including their interaction terms) were specified as fixed effects, and population nested within range as a random effect. These models were fit by REML estimation in the 'lme4' package (Bates et al., 2015) used in conjunction with 'lmerTest' (Kuznetsova et al., 2015), which provided estimates of parameter significance by means of a Satterthwaite approximation. Model simplification was then performed, first through assessment of multicolinearity by variance inflation

factors (VIFs), followed by removal of non-significant covariates (at an α-level of 0.05). Significant interactions in final models were explored through post-hoc interaction analysis in the package 'phia' (Martınez, 2015). Finally, R^2 values were generated according to Nakagawa and Schielzeth (2013), for estimation of marginal and conditional R^2 for fixed effects, and total (i.e. both fixed and random) effects, respectively. Models were validated through statistical and graphical assessment of standardised residuals for normality, homogeneity of variances and spatial independence (Zuur et al., 2009). Additionally, where significant fixed effects were indicated, a likelihood-ratio test was employed to test that AIC (Akaike Information Criterion) of the mixed model was significantly lower than that of a null model containing random effects only.

Climatic and environmental variables found to explain significant variation in the above conducted LMMs were further explored by means of a partial Mantel test, which was conducted at n = 1000 permutations in the package 'ecodist' (Goslee and Urban, 2007). This test assessed whether the relationship between nectar GTX levels and the variable of interest held after controlling for geographic distance between plants (based on coordinates recorded in the field using a hand-held GPS to an accuracy of ca. 5 m). To assess how potential fluctuation in environmental conditions between years may influence the levels of GTX in nectar, a paired t-test was carried out, examining whether mean nectar GTX I and GTX III levels differed in plants between years.

In addition to quantitative comparisons between ranges, geographic variation in nectar toxin composition was assessed on a qualitative basis, as chemical analyses indicated a dichotomy in toxin profiles according to the dominant GTX in nectar. Plants were hence divided into two chemotypes; those which produced abundant GTX I as the main nectar toxin were designated as Chemotype A, and those which exhibited GTX III as the predominant nectar toxin (with little or no detectable GTX I) classified as Chemotype B. The geographic distribution of these chemotypes was subsequently examined within and between the native (Spain and Portugal) and introduced range.

Spatial structure

To examine potential spatial structure in plant nectar GTX I levels, correlogram analyses were used to measure spatial autocorrelation (or the extent to which GTX levels were aggregated) at patch and landscape scale in each range. We here define a patch as a cluster of plants within a ca. 100 m neighbourhood, given the typical dispersal distances of *R. ponticum* which influence population structure (Stephenson et al., 2007). Correlograms were generated in the 'vegan' package (Oksanen et al., 2013) at distance lags of 24 m for patch-scale analyses, and distance lags ranging between 5 and 130 km for landscape-scale analyses. It was necessary to set the latter distance lags at irregular intervals due to the naturally sporadic distances between populations sampled in the field. Significance values reported were subject to Holm's progressive correction for multiple-testing (*n* = 1000 permutations).

Results

Geographic variation and spatial structure

Linear mixed models indicated a significant effect of range on nectar GTX I levels (Table 1); GTX I levels were estimated to be on average 1.8 times higher in the native range in Spain (mean GTX I = 1.46 µg/mg ± 0.10 SE) in comparison to the introduced range in Ireland (0.81 µg/mg ± 0.08 SE) (Fig. 1). In contrast, nectar GTX III levels were not significantly different in the native and introduced ranges (Table 1, Fig. 1). Within ranges, significant population variation was observed for both GTXs (Table 1), with on average five to six-fold differences between plants that produced the lowest and highest levels of each GTX within populations. Nectar water content did not differ between ranges, but exhibited significant population variation within ranges (Table S3). On a qualitative basis, a unique chemotype was evident in the introduced range which possessed GTX III (as opposed to GTX I) as the main nectar GTX (Fig. 2). This chemotype (i.e. Chemotype B) constituted 22 % of plants in the introduced range (*n* = 50); with 18% of plants in the introduced range devoid of GTX I in nectar. The majority of introduced populations were

found to contain Chemotype B, and it constituted the major chemotype in one. In contrast, Chemotype B was absent amongst all plants (n = 50) and populations examined in the native range (Fig. 2).

Correlogram analysis examining spatial structure in nectar GTX I levels revealed distinct patterns of autocorrelation in the native and introduced range of *R. ponticum* (Fig. 3). Spatial autocorrelation was detected at patch scale among plants located up to 70m from each other (Fig. 3a), as well as at landscape scale among populations located within 15-23km of each other (Fig. 3b). Thus only plants/populations which occurred beyond these distances were deemed spatially independent. The shape of correlograms indicated an irregular pattern of spatial aggregation in the introduced range at both scales, in comparison to the native range, where change in nectar GTX I levels was more gradual across patches and landscapes.

Environmental variation

Large-scale climatic variables which were spatially heterogeneous across ranges (such as precipitation, temperature etc.) did not account for significant variation in LMMs for GTX I or GTX III (Table 1). On the other hand, a positive association was observed between nectar GTX I levels and the amount of heat load intercepted by plants (a function of latitude, aspect and slope), which varied across patch and landscape scales. However, this relationship was not constant across ranges (as indicated by the significant interaction in Table 1), as heat load exhibited a much stronger effect on nectar GTX I levels in the introduced range (slope = 0.0055, χ^2 = 19.2, p < 0.001) than in the native range (slope = 0.0007, χ^2 = 0.2, p = 0.629). While the amount of intercepted heat load is expected to be similar for neighbouring plants in populations, this relationship also held across populations in the introduced range; even after controlling for geographic distance, plants which grew in similar heat load environments tended to possess similar nectar GTX I levels (Partial Mantel test: r = 0.28, P < 0.001). The potential inter-annual fluctuation of environmental conditions did not appear to be a relevant factor affecting nectar toxin

levels, as no significant variation in nectar GTX I and GTX III levels was observed in plants across years (Paired *t*-test: $t = -1.49$, $P = 0.180$; $t = -1.16$, $P = 0.285$).

Discussion

In this study, we demonstrated considerable quantitative and qualitative intraspecific variation in toxic nectar, within and between the native and introduced range of *Rhododendron ponticum*. This complements studies which have shown geographical variation in secondary compounds as a common feature in leaves and other plant parts (Berenbaum and Zangerl, 2006, Harborne, 1986, Hunter et al., 1996). While qualitative variation in nectar cardiac glycosides has previously been demonstrated amongst closely-related species of milkweed (Manson et al., 2012), this study is the first to show marked within-species differences in the qualitative composition of toxic nectar. Geographic variation in nectar GTXs was compound-specific, however. Nectar GTX I varied both across ranges, and between populations within ranges, whereas only population variation in nectar GTX III was evident. Furthermore, phenotypic expression of nectar GTX I was spatially structured at patch and landscape scales. Few studies have examined how nectar traits are structured in a spatial context, despite evidence that autocorrelation at the patch level may facilitate the efficacy by which pollinators forage on plants displaying desirable nectar qualities (Leiss and Klinkhamer, 2005).

When present in the nectar of plant species, toxic compounds are typically found at low or trace levels relative to other plant parts. In contrast, we found nectar GTX I levels in native plants at levels up to 5.67 μg/mg (or 4110 μg/ml); this concentration is half that found in the leaves and fresh twigs of this species (total GTX 9.28 μg/mg and 15.90 μg/mg, respectively (Hough et al., 2010, Wong et al., 2002)), and is one of the highest known in plant species to-date (Adler and Irwin, 2005, Wright et al., 2013, Adler and Irwin, 2012, Boros et al., 2010, Cook et al., 2013, Gosselin et al., 2013, London-Shafir et al., 2003, Manson et al., 2012). Furthermore, the levels of GTX I found in Spainish *R. ponticum* nectar are

significantly more concentrated than as found in 'mad honey' (GTX I 0.02 μg/mg), which is derived from flowers of the Turkish/Caucasian subspecies *R. ponticum* subsp. *ponticum* (Kurtoglu et al., 2014).

The biological activity of toxic compounds in nectar is almost certainly dose-dependent (Lerch-Henning and Nicolson, 2013, Manson et al., 2013, Tadmor-Melamed et al., 2004). For this reason, both the extent of spatial variation in toxic nectar compounds, and how this variation is structured in space could hold a substantial impact on the ecology of plant-pollinator mutualisms in *R. ponticum* and other plant species. In our study, for example, the occurrence of chemotypes which exhibit little to no GTX I in nectar and spatial aggregation of low GTX-producing plants may facilitate visitation by pollinators sensitive to this toxicity. Although individuals of *Bombus terrestris* appear unaffected by average levels of nectar GTX I and III (Tiedeken et al., 2016), it is not known how tolerant *B. terrestris* is of higher values of these compounds in the introduced range, or GTX I levels in the native range ca. double this concentration. Other species including honeybees and a solitary mining bee (*Andrena* Fab.) experience dramatic lethal and sublethal effects from consuming average levels of GTX I, but not GTX III, in the introduced range (Tiedeken et al., 2016). Yet some *Andrena* species are floral visitors of *R. ponticum* here (Stout, 2007a), and may thus favour visitation to plants showing lower levels of nectar GTX I – which could be discriminated on a spatial basis. Similarly, both the extent and spatial structure of variation in nectar toxins could modulate food quality and host choice by floral larcenists of *R. ponticum*. In both ranges these include multiple ant species (Haverkamp, 2011, Herrera et al., 1984), and pollen beetles such as *Meligethes aeneus* Fab. – although the latter may not be purely antagonistic, as they can carry a small amount of *R. ponticum* pollen adhered to their bodies (Mejias et al., 2002). It remains unknown, however, if nectar GTXs impact such antagonists, and whether plants which exhibit diminished nectar defences are consequently more prone to exploitation.

In addition to biotic drivers, abiotic environmental conditions are known to regulate secondary metabolite production in plants at a range of spatial scales (Hunter et al., 1996, Laitinen et al., 2005, Moore and DeGabriel, 2012). In examining potential climatic and environmental influences on toxic nectar, the amount of heat load intercepted by plants was significantly associated with nectar GTX I levels in the introduced range. While strongly related to the level of irradiance intercepted, heat load reaches a maximum in plants exposed to steeply sloping south-western as opposed to southern aspects (McCune and Keon, 2002), and is often locally and regionally heterogeneous. High irradiance and temperature regimes have been known to induce diterpene production in some plant species, as a plastic response to stress conditions (Bertolucci et al., 2013, Munne-Bosch et al., 1999). It is thus conceivable that increased levels of GTX I in nectar arise from a similar physiological stress-response in plants, most probably originating in leaves, given the main foliar-plastid origin of terpenoid biosynthesis (McGarvey and Croteau, 1995).

The majority of climatic and environmental variables examined in this study were not associated with variation in nectar GTX levels. This potential constancy in phenotypic expression was corroborated by the finding that nectar GTX levels did not significantly differ in plant individuals between years, in spite of possible fluctuation in biotic and abiotic conditions. In contrast, environmentally-induced variation in toxic nectar has previously been reported in response to plant nutrient status (Adler et al., 2006). Although based on an inter-annual comparison from a single population, these findings could suggest an overall robustness of nectar toxin levels to environmental heterogeneity. Given the dynamic and highly variable nature of many nectar traits in plant species (Canto et al., 2011, Mitchell, 2004), this potential phenotypic constancy is notable. The ability for individuals to maintain tightly regulated levels of nectar toxin expression in the face of environmental heterogeneity may be particularly critical given the possible ecological costs of maladaptive (or ‘injurious’) plasticity in this trait. The topic of temporal

variation would hence benefit from future assessments which consider both finer and coarser time scales and which sample across multiple populations.

Although most of the climatic and environmental variables measured in this study did not account for significant variation in nectar GTX levels, it is nonetheless possible that other unmeasured variables could have contributed towards the quantitative differences observed within and between ranges. Regardless of this fact, it is difficult to explain the qualitative absence of GTX I in nectar (observed in 18 % of introduced plants dispersed across most populations; Fig. 3) as being due to phenotypic plasticity. Random genetic drift and founder effects also do not appear likely given; A.) the magnitude of change observed for nectar GTX I (whereas GTX III showed no difference between ranges); and B.) that introduced populations in Ireland are known to retain levels of genetic and morphological diversity comparable to that found in the ancestral range (Erfmeier and Bruelheide, 2011, Stout et al., 2015).

As a genetic-based shift in other functional traits has prospectively occurred in *R. ponticum* in the time since its first introduction in Ireland (Erfmeier and Bruelheide, 2005, Stout et al., 2015), adaptation to biotic pressures could likewise explain why GTX I expression is markedly lower or absent from nectar in introduced plants. There are considerable metabolic costs to the production of plant terpenoids (Gershenzon, 1994), and thus enemy-release from floral antagonists such as nectar robbers, or pollinator-mediated selection against high nectar GTX I (which is differentially more toxic than GTX III to some pollinators), are consistent with the changes in nectar phenotype observed in this study. Alternatively, given that the defensive chemistry of leaves and nectar may be linked within plant individuals (Adler et al., 2006), reduced nectar toxin levels could also stem from decreased plant allocation to foliar defence, arising from a change in herbivory pressure (as per the 'evolution of increased competitive ability' (EICA) hypothesis (Joshi and Vrieling, 2005)). However, introduced populations of *R. ponticum* have not experienced a major evolutionary release from foliar herbivory, as

is predicted by EICA. Introduced populations in fact show a well-developed herbivore fauna (Judd and Rotherham, 1992, Yela and Lawton, 1997), and a majority of individuals exhibit at least minor to moderate leaf damage (PA Egan, personal observation). It is therefore unlikely that foliar interactions are ultimately responsible for the patterns observed in this study for nectar GTXs.

Prominent hypotheses which implicate plant defence as an important component influencing the success of introduced species, such as EICA and the related concepts of 'enemy-release', 'novel weapons' and 'shifting defence' (Callaway and Ridenour, 2004, Cappuccino and Arnason, 2006, Doorduin and Vrieling, 2011, Joshi and Vrieling, 2005), have been implicitly formulated in relation to plant vegetative parts, and their extension to the defensive chemistry of nectar and other mutualist rewards is not straight-forward. In contrast to other plant parts, floral nectar has primarily evolved to be consumed by pollinating mutualists. Thus, changes in defence allocation in mutualist rewards may differ substantially from as is predicted for tissues exploited solely by herbivores. The findings of this study, coupled with a previous investigation in this system (Tiedeken et al., 2016), therefore suggest that toxic nectar in introduced species should bear an overall negative effect on floral mutualists, and that selection should act against these compounds (although natural selection on toxic nectar has yet to be empirically demonstrated for any species). Based on our findings, future development of invasion theory should progress beyond a predominant focus on antagonistic interactions, and seek to integrate mutualisms into a predictive framework of plant defence.

Acknowledgements

We acknowledge Juan Arroyo and Mark Brown for their kind input and facilitation of the research, and Dirk Holstege for providing a reference standard of grayanotoxin I. This work was funded by Science Foundation Ireland (10/RFP/EOB2842, to J.S.). G.A.W. and P.S. were funded jointly by a grant from

BBSRC, NERC, the Wellcome Trust, Defra, and the Scottish Government under the Insect Pollinators Initiative (BB/I000143/1). E.J.T. was funded by the Irish Research Council (RS/2010/2147).

Data Accessibility

- Sampling locations and characteristics of *Rhododendron ponticum* populations: uploaded as online supporting information
- Data file containing individual plant coordinates, nectar GTX concentration and climatic and environmental covariates: DRYAD entry doi:10.5061/dryad.6p46m / figshare entry doi: https://dx.doi.org/10.6084/m9.figshare.3101104.v1

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SUPPORTING INFORMATION

Table S1 Sampling locations and characteristics of *Rhododendron ponticum* populations

Table S2 Climatic and environmental variables quantified per plant of *Rhododendron ponticum*

Table S3 Linear mixed models analysing the effect of range (i.e. native versus introduced) on nectar water content in *Rhododendron ponticum*

Table 1. Linear mixed models analysing the effect of range (i.e. native versus introduced) and environmental covariates on nectar grayanotoxin (GTX) I and III levels in *Rhododendron ponticum*. Population nested within range was included as a random factor. Marginal and conditional R^2 values indicate the variance explained by fixed effects and both fixed and random effects, respectively

Model	GTX I			GTX III		
	F / χ^2 (df)	Sig.	R^2	F / χ^2 (df)	Sig.	R^2
Fixed effects			0.44			0.00
Range	$F_{(1, 10)} = 24.2^{\dagger}$	***		$F_{(1, 11)} = 0.004$	ns	
Heat load	$F_{(1, 88)} = 9.9$	**				
Range x Heat load	$F_{(1, 88)} = 5.8$	*				
Random effects						
Population(Range)	$\chi^2_{(1)} = 8.2$	**		$\chi^2_{(1)} = 15.9$	***	
Total model			0.58			0.33

[†] A range effect was estimated based on the mean value of heat load. A significant effect remained in addition at the 10th ($\chi^2 = 28.0$, $P < 0.001$) and 90th ($\chi^2 = 4.0$, $P = 0.046$) percentile of this covariate

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant

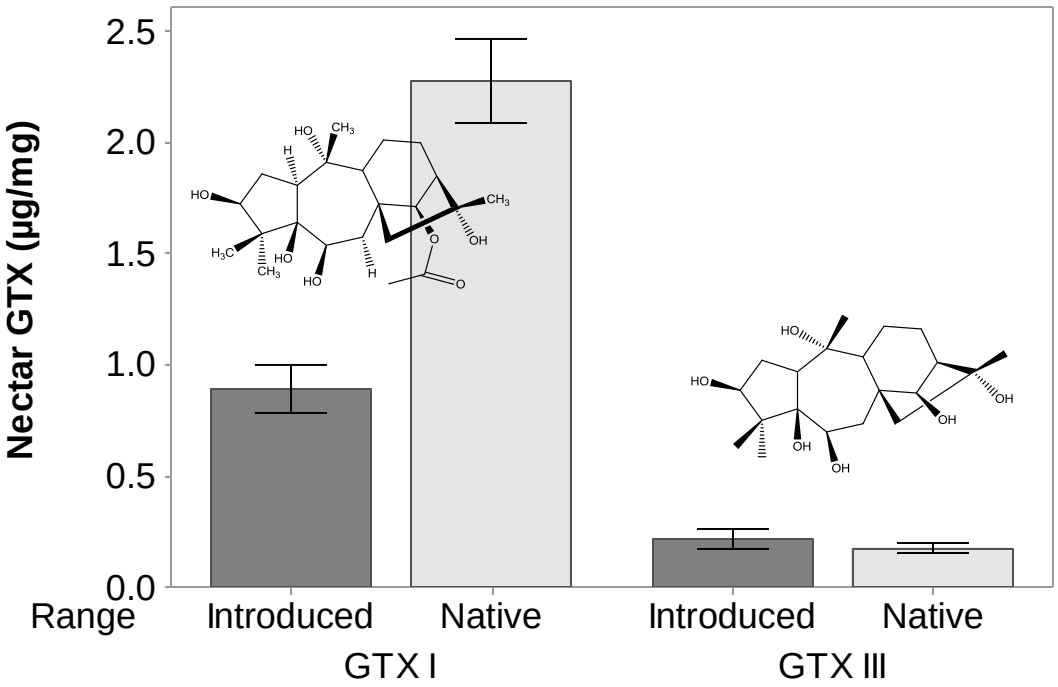

Figure 1. Comparison of nectar grayanotoxin (GTX) levels (mean ± SE; untransformed data) between the introduced and native range of *Rhododendron ponticum* (*n* = 50 plants per range). Test results from linear mixed models are presented in Table 1. The chemical structure of each GTX is depicted.

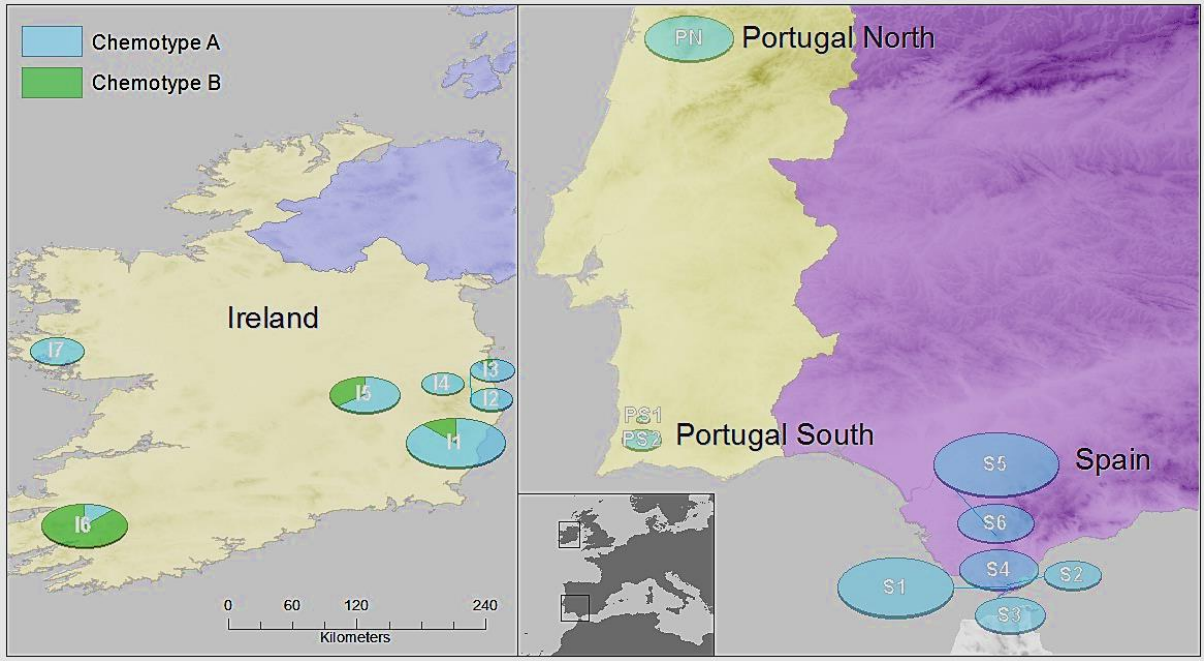

Figure 2. Geographic distribution of nectar chemotypes of *Rhododendron ponticum* within and between the native and introduced range. The proportion of plants exhibiting Chemotype A (GTX I-dominant) and Chemotype B (GTX III-dominant) is depicted per population, with pie size proportional to mean grayanotoxin levels. Population codes and sample sizes correspond to as listed in Table S1.

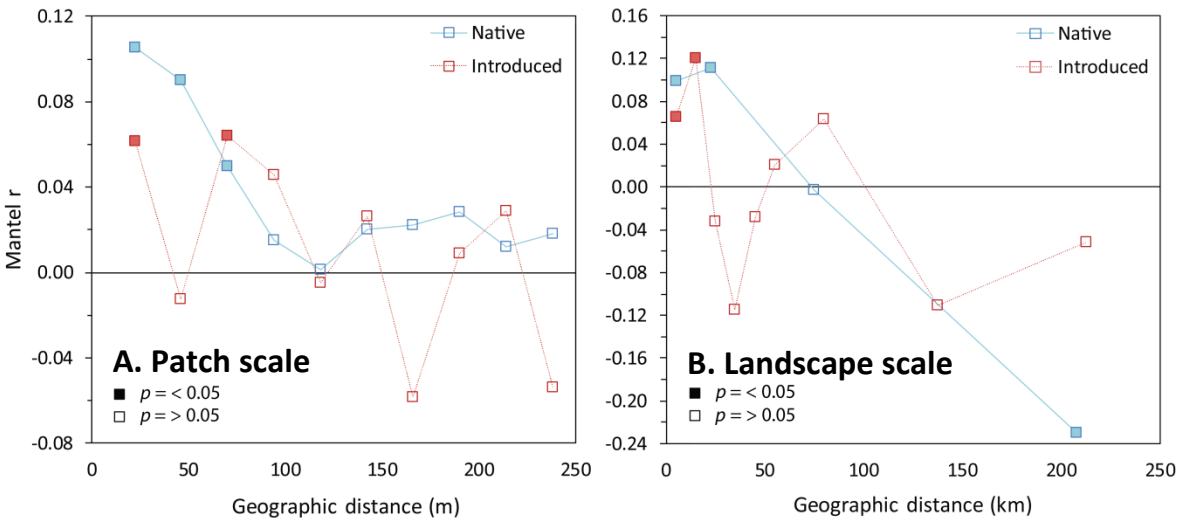

Figure 3. Correlogram analysis of nectar grayanotoxin I levels in *Rhododendron ponticum*. Significant autocorrelation among plants was evident in both the native and introduced range at (**a**) patch scale within populations up to 70 m, and (**b**) landscape scale between populations up to 15-23 km (*n* = 71 native plants, 50 introduced plants). Spatial autocorrelation was absent or negative beyond these distances (with points close to the zero-line indicating a non-significant Mantel *r*).