Nectar chemistry modulates the impact of an invasive plant on native pollinators

Running headline: Impacts of toxic nectar on three pollinators

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Summary

1. Invasive species are considered a main driver of pollinator declines, yet the direct effects of invasive alien plants on pollinators are poorly understood.

2. Abundant, invasive plant species can provide a copious nectar resource for native pollinators. However, the nectar of some plants contains secondary compounds, usually associated with defence against herbivores. The impacts of these compounds on pollinators are often unknown.

3. We compared how consumption of grayanotoxin I and III, natural secondary compounds in the nectar of invasive *Rhododendron ponticum* L., affected three native bee species: a honeybee, (*Apis mellifera* L.), a solitary mining bee (*Andrena carantonica,* Pérez) and a bumblebee, (*Bombus terrestris,* L.).

4. Survival of the solitary bee and the bumblebee species was not affected by either grayanotoxin, but honeybees were ~20x more likely to die when fed solutions containing grayanotoxin I. Furthermore, solitary bees were deterred from feeding and exhibited malaise behaviours indicative of sublethal toxicity in response to consumption of grayanotoxin I. In contrast, grayanotoxins did not affect bumblebee survival or behaviour, even when bees were subjected to multiple stressors (parasite infection or food stress).

5. Our experiments suggest that while *R. ponticum* provides abundant floral nectar, it is only available as a food resource to pollinators that tolerate grayanotoxins. Pollinators whose health is negatively affected by grayanotoxins may experience negative impacts from *R. ponticum* invasion directly (if they consume *R. ponticum* nectar) or indirectly (if native floral resources are replaced by *R. ponticum*).

6. Our study makes a novel comparison of the effects of a natural nectar secondary compound on three pollinator species and clearly demonstrates drastic variation in the responses of different key pollinator taxa to a nectar toxin. Our findings are thus in congruence with literature demonstrating the varying effects of invasive plant chemistry on native foliar

herbivores and our work demonstrates that nectar chemistry should be taken into account when determining the impacts of plant invasion for native pollinators.

Key words: invasive alien plants, multiple stressors, *Rhododendron ponticum*, secondary compounds, toxic nectar

Introduction

Invasive species are considered a key driver of pollinator decline (Gonzalez-Varo *et al.* 2013), yet little research has investigated the direct impacts of invasive plants on native pollinators (Stout & Morales 2009). The direction of impacts will depend on how plant invasion influences the availability of resources essential to pollinators, for example forage resources. Invasive plant species could reduce nectar and pollen resources for pollinators when they outcompete native plant species (Cox & Elmqvist 2000; but see Sax *et al.* 2007), eventually leading to changes in pollinator community structure (Aizen, Morales & Morales 2008). Conversely, entomophilous, mass-flowering invasive plants may provide pollinators with abundant nectar and pollen, especially in areas with few native flowers (Graves & Shapiro 2003). This could mitigate the loss of native flowering plants, and may even increase pollinator carrying capacity (Tepedino, Bradley & Griswold 2008).

However, it is not just the abundance of floral rewards offered by invasive species that may influence native pollinators, but reward quality as well (Stout & Morales 2009). Some floral nectar is known to be toxic or unpalatable to pollinators (Pryce-Jones 1942; Majak, Neufeld & Corner 1980) due to the presence of secondary compounds; these compounds are usually associated with defence against foliar herbivory, for example, alkaloids, terpenes, or phenolics (Adler 2000). Nectar secondary compounds are geographically and phylogenetically widespread (Adler 2000), however, their impacts on pollinators are often poorly understood (Cook *et al.* 2013; Manson *et al.* 2013).

Previous work has demonstrated pollinator responses to nectar secondary compounds from native plants that range from positive to negative (Detzel & Wink 1993; Manson, Otterstatter & Thomson 2010). Nectar secondary compounds tend to occur at low concentrations (Adler & Irwin 2012) which rarely have acute lethal effects for pollinators (but see (Pryce-Jones 1942; Majak, Neufeld & Corner 1980)). However, sublethal effects could result in decreased growth or fecundity for pollinator individuals and/or colonies (Desneux, Decourtye & Delpuech 2007). Consumption of nectar secondary compounds can affect pollinator physiology (Manson & Thomson 2009), behaviour (Wright *et al.* 2010; Cook *et al.* 2013; Manson *et al.* 2013; Wright *et al.* 2013) and subsequently fitness, but impacts are often dose-dependent and may only be apparent at unnaturally high concentrations (Tiedeken *et al.* 2014). In addition, pollinators are simultaneously exposed to multiple stressors, including parasite infection and food stress (Gonzalez-Varo *et al.* 2013; Goulson *et al.* 2015). When combined with additional stressors, negative impacts of consumption of nectar secondary compounds on pollinator health may be realized (Brown, Loosli & Schmid-Hempel 2000; Holmstrup *et al.* 2010). While the impacts of multiple stressors on pollinators are recognized as causing potentially additive or synergistic effects (Vanbergen & Initative 2013), few studies have addressed this issue.

The aim of the present study was to investigate how nectar secondary compounds from an abundant, invasive plant species impact native pollinators. We focused on impacts on native bees because they are ecologically and economically important pollinators (Morse & Calderone 2000; Garibaldi *et al.* 2013) but in decline worldwide (Biesmeijer *et al.* 2006). Using a series of laboratory-based, non-choice bioassays we tested the following hypotheses:

1. Nectar secondary compounds from an invasive species have lethal effects on native bees.
2. In the absence of lethal effects, nectar secondary compounds from an invasive species cause sub-lethal changes in bee behaviour and food consumption.

3. Nectar secondary compounds exacerbate the effects of parasite infection and food deprivation in bees.

Materials and Methods

Study system

Invasive *Rhododendron ponticum* subsp. *baeticum* was introduced from the Iberian peninsula into Britain and Ireland in the eighteenth century (Cross 1975). Mature plants produce hundreds of flowers containing copious volumes of sugar-rich nectar, making plants attractive to native insects, particularly bees (Stout *et al.* 2006) which act as the main pollinators (Stout 2007a). Despite its reliance on insects for pollination (Stout 2007b), the nectar of *R. ponticum* contains high concentrations of diterpenes known as grayanotoxins (GTXs) (Tiedeken *et al.* 2014). *R. ponticum* nectar contains GTX I and III, but GTX I is quantitatively dominant (personal observation P. Stevenson). GTXs are known for their toxicity to mammals (Gunduz *et al.* 2008), and can negatively affect herbivore physiology and behaviour (El-Naggar *et al.* 1980; Klocke *et al.* 1991), but little is known of their toxicity to pollinators, including bees.

Artificial nectar preparation

In total 48 ml of floral nectar was collected from approximately 5,400 *R. ponticum* flowers from four populations in Ireland (Table S1), and analysed for sugar content and GTX concentration (Appendix S1 and Fig. S1 in Supporting Information). The total GTX content (GTX I and III) of the pooled nectar was 0.44 μg GTX per milligram fresh weight nectar (determined using methodology described in Tiedeken *et al.* (2014)). GTX I was isolated from *R. ponticum* floral material collected from Irish populations (Appendix S1, Table S1), because it is not commercially available. GTX III was purchased from Sigma-Aldrich, Dublin, Ireland. Five treatment solutions were used in assays, but not all treatments could be used in every assay because a.) GTX I supplies were limited and b.) the availability of bees differed by species.

Treatment 1 was *R. ponticum* nectar extracted from wild-growing flowers; treatment 2 was an artificial nectar that contained no GTX but simulated *R. ponticum* nectar sugar content; treatment 3 was the same artificial nectar but contained GTX I and GTX III at the natural ratios found in *R. ponticum* nectar; treatment 4 was the artificial nectar that contained natural concentrations of only GTX I; and treatment 5 was the artificial nectar that contained natural concentrations of only GTX III (see Table 1 for more details). The different treatments were utilized in order to determine the biological activity of GTX I, III and the two combined compounds (Table 1).

We mixed sucrose, fructose, and glucose (Sigma-Aldrich) with deionized water to obtain a base solution simulating the sugar concentration of *R. ponticum* nectar (Fig. SI1). The base solution was warmed (<50°C) and GTXs were added to create treatment solutions. All solutions were prepared and immediately stored at -80 °C until ready for use. Samples of final solutions were analysed to verify GTX concentrations.

Bee species

We used three bee species that are native to habitats invaded by *R. ponticum*; the honeybee, *Apis mellifera mellifera* (the European Dark Honeybee), a bumblebee species, *Bombus terrestris audax* (the buff-tailed bumblebee), and a solitary mining bee, *Andrena carantonica*. These species and subspecies were chosen because they are native to Britain and Ireland, where *R. ponticum* is an invasive species. In 2012, honeybees were obtained from two queen-right free foraging, disease-free colonies, from Irish-reared queens at the Trinity College Dublin Botanic Gardens. Bumblebee colonies were obtained from a commercial supplier (Unichem, Ireland, who source the native subspecies *B. terrestris audax* from Koppert Biological Control, The Netherlands), and upon arrival were queen-right, still producing worker brood, and screened for parasites by examining faecal samples from 10 workers per colony. Female *A. carantonica* individuals were collected from an aggregation on a south-facing incline at Trinity

College Dublin in spring, 2013. Individuals returning to their nests after foraging were collected on warm days (>15 °C) and brought back to the lab to acclimate before being used in the study. No ethical approval or licenses are required at the State or University level for insect bioassays, but we complied with good research practices throughout the study.

Survival assays

Honeybees

Two hundred and fifty honeybees were collected in plastic vials (2.5 cm diameter) at the hive entrances as they returned from foraging. Individuals were chilled on ice until movement ceased, weighed, and restrained using plastic harnesses (Bitterman *et al.* 1983). Harnessed bees were immediately fed 5 μL 50% Apiinvert solution (inverted sugar solution provided to supplement the diet of commercial bees, from Bee Supplies, Sandyford, Dublin), allowed to acclimate for 1 h and then fed 4 additional 5 μL Apiinvert drops. Bees showing an unreliable proboscis extension response (PER) were excluded from experiments. Bees were left overnight in climate controlled chambers (Adaptis, Conviron ^{TM}) at 25°C, 70% relative humidity, 0 light. The next morning, 50 bees were randomly assigned to one of five treatments (Table 1) and fed 5 x 5 μL drops of treatment solution. Bees were monitored hourly for 6 h to track survival. This process continued until 50 bees, 25 from each colony, were fed each treatment.

Oral toxicity tests were also conducted for the acute toxicity testing of honeybees (methodology in Appendix S2).

Bumblebees and solitary bees

To directly compare bumblebee and honeybee responses to GTX, an identical assay with restrained bumblebees was performed (methodology in Appendix S3). Long-term assays were also carried out with unrestrained bumblebees to investigate chronic effects. Workers from each of three *B. terrestris* colonies were weighed, and randomly allocated to one of the five treatments (Table 1). Bees were placed individually into 650 ml plastic containers

(160x110x45 mm) with lids containing ventilation holes (1 mm diameter). A 10 mm diameter hole was located on the side of the container where feeding tubes (0.75 ml centrifuge tubes with four 1.5 mm holes) could be inserted horizontally. Bees could alight on the feeding tube. A dish (0.5 cm diameter) containing pollen (3.2 g ± 0.34 g) (Koppert Biological Systems) was provided on day one. All five treatments (Table 1) were fed to the bees for seven days (n= 6 bees for treatment 1, n=12 bees for treatments 2-5). Because the availability of treatment solutions was limited, only the control treatment (treatment 2) and the treatment containing GTX I and III (treatment 3) were fed to bees over a 30 day period (n=12), the approximate flowering time of *R. ponticum* (Stout 2007b). Bees were kept in a growth cabinet (Adaptis, Conviron ™) at 28°C, 60% RH, and 12h:12h dark/light. Survival was recorded and treatment solutions were replaced daily.

Because we had a limited number of individuals, we carried out the *A. carantonica* assay with two treatments on unrestrained bees (n = 18 bees per treatment). This assay was identical to the unrestrained bumblebee assay described above. Bees were randomly assigned to either the control treatment (treatment 2) or the treatment containing GTX I and III (treatment 3). Bees were fed 50% Apiinvert solution *ad libitum* during the first 24 h and were kept in a growth cabinet (20 °C, 60% relative humidity, 12:12 dark/light setting) throughout the experiment. Survival was recorded daily for 30 days.

Sublethal effects

Because honeybees were harnessed and demonstrated an acute lethal response to nectar GTXs (Fig. 1), behavioural responses were not measured.

In order to record differences in the response of bumble or solitary bees fed GTX, behaviour was monitored continuously for 90 s per bee per day, on 11 days throughout the unrestrained survival assays. Seven distinct behaviours were observed (see Appendix S4). The amount of treatment solution consumed by bumblebees and solitary bees during the 30-day unrestrained

assays was also recorded. Feeding tubes were weighed initially and after 24 h to record daily consumption (grams) and external controls were used to account for evaporation.

Additional stressors: Parasite and survival under stress assay

Bumblebees were used in both additional stressors assays because they did not exhibit any lethal or sub-lethal effects and are the main pollinators of *R. ponticum* in its invasive range (Stout 2007a).

First we investigated how GTX consumption impacted infection with a common parasite. A *Crithidia bombi* inoculum was created for three *B. terrestris* colonies (Koppert) by harvesting faecal samples from workers previously infected with *Crithidia* from wild-caught queens (as in Brown, Loosli & Scmid-Hempel (2000), see Appendix S5). Sixty workers per colony were infected with their colony-specific *Crithidia* inoculum. The workers were randomly divided into two groups, kept individually (as per the survival assay), and fed either the control treatment (treatment 2) or the treatment containing GTX I and III (treatment 3) for the next 10 days, until the parasite load was at its peak. On day 10, a final faecal sample was collected from each bee, diluted 10 fold with Ringer's solution (Sigma-Aldrich), and *Crithidia* load was determined using haemocytometer counts. In addition, a standard starvation assay (survival under stress) was carried out (see Appendix S6 for methodology and results).

Data analysis

Survival data were analysed using Cox regression proportional-hazards models in the survival package in R (Therneau & Grambsch 2000; R Core Team 2015; Therneau 2015). For honey and bumble bees, we controlled for a colony effect by including a frailty function in the models. Individual bee weight was originally included in survival models, but was removed in the final analyses because it was not a significant factor. Because survival models cannot run on completely censored data, we changed the status of one individual in the honeybee assay, treatment 5, on the last day of the experiments to "dead," then modelled the unaltered data with

Kaplan-Meier survival analysis with log-rank tests to verify the robustness of this method (Tragust *et al.* 2013). Dose response data for the honeybees were analysed using a logit regression model in SPSS Statistics (version 19). Mortality was less than 20% in all control groups, thereby meeting the requirements of the USEPA's ecological effects test guidelines (1996).

The total proportion of time an individual spent performing each behaviour in the control and GTX treatments was compared using a Mann Whitney U test. For bumble and solitary bee consumption data, the daily average consumption was calculated for each individual and compared between the two treatments using a Mann Whitney U test. Time was excluded as a factor in the consumption analysis because for the solitary bees, the number of dead bees increased considerably throughout the course of the experiment, significantly impacting the fit of the model. Consumption results therefore cannot compare how short-term vs. long-term exposure impacts feeding behaviour, however they give an overall idea of differences in consumption between the bumble and solitary bee species. Consumption data and parasite loads for the bumblebee multiple stressors assays were analysed using linear mixed effects models, with treatment as a fixed factor and colony as a random factor in the nlme package in R (Pinheiro *et al.* 2015). Again, individual bee weight was excluded from the model because it was not a significant factor. Parasite load was log transformed in order to meet the assumptions of normality.

Results

Survival assays

Within six hours after consumption, honeybees exhibited an acute lethal response to *R. ponticum* nectar (Fig. 1a). All treatments containing GTX I increased mortality compared to the control. In contrast, treatment with the solution containing only GTX III was not

significantly different to the control (χ_4^2= 150.8, P < 0.001, Fig. 1a). Honeybees fed *R. ponticum* nectar (treatment 1) had a 12-fold increased risk of death (Hazard ratio (HR) = 12.1, P < 0.001), whilst bees fed the treatment solutions that contained GTX I (treatments 3 and 4) had a 21-fold increased risk of death (HR= 21.0, P < 0.001). After correcting for multiple testing, there was no significant difference in mortality between honeybees fed *R. ponticum* nectar and those fed treatments with only GTX I. The random factor colony had no impact on survival (P = 0.920). Honeybees in the control treatment of the oral toxicity assay had low mortality, 3.3% at 24 h and 6.7% at 48 h. In contrast, individuals fed the naturally occurring concentration of GTX I (0.44 µg/mg) experienced 73.3% mortality at 24 h and 76.7% at 48 h. The 24 h LC_{50} for GTX I was 0.212 µg/mg for honeybees, approximately half the natural concentration found in *R. ponticum* nectar. The value for the 48 h LC_{50} was lower still, 0.172 µg/mg (Table S2).

In contrast to the honeybees, consumption of GTXs did not cause an acute lethal response in bumblebees. In the unrestrained seven-day assay comparing all five treatments, only one individual died in each treatment, except for bumblebees fed *R. ponticum* nectar (treatment 1) in which no deaths were recorded (Fig. 1b). In the 30-day assay comparing the control treatment (treatment 2) with GTX I and III (treatment 3), no bumblebees in either treatment died (Fig. 1c.). In the 24 h harnessed assay, no bumblebees in any of the five treatments died in the six-hour period after they were fed.

There was an initial die-off of solitary bees in both treatment groups but the death rate stabilized around day five. At the end of the experiment 84.2% of the control solitary bees (treatment 2) and 88.9% of the solitary bees fed GTX I and III (treatment 3) died (Fig. 1d.). Survival analysis indicated that treatment had no significant effect on survival (likelihood ratio test: χ_1^2= 0.3, P = 0.583).

Sublethal effects

Treatment did not have a significant effect on any bumblebee behaviour (Table 2). Solitary bees fed GTX I and III (treatment 3) exhibited excessive grooming or paralysis behaviours for a significantly higher proportion of time than solitary bees fed the control (treatment 2, Mann Whitney U test, W = 81, $P < 0.001$). Control-fed solitary bees never demonstrated excessive grooming or paralysis, and spent a significantly higher proportion of time flying than GTX-fed solitary bees (W = 228, $P = 0.006$).

Bumblebees consumed on average 0.293 ± 0.019 g solution daily but there was no significant effect of treatment on consumption (W = 75.0, $P = 0.887$, Fig. 2a). Overall, solitary bees consumed less than bumblebees (solitary bee daily mean = 0.0357 g ± 0.002), and solitary bees fed the control solution consumed on average double that of solitary bees fed the GTX solution (W = 254.5, $P = 0.011$, Fig. 2b.).

Additional stressors

In the parasite assay, all bumblebees were infected with *C. bombi* at day 12 except two individuals, which were excluded from the analysis (assumed parasite free due to experimental error). Bees experienced 33.9% and 32.2% mortality in the control treatment (2) and the GTX I and III treatment (3) respectively. There was no significant effect of treatment or the random factor colony on survival (Fig. 3a. $\chi_1^2 = 0.57$, $P = 0.508$). At peak infection, the parasite load of the bees fed the GTX I and III treatment were on average slightly higher than those fed the control treatment (Fig. 3b.), however, this difference was not significant ($F_{(1,2)} = 0.240$, $P = 0.672$) and there were no differences among colonies ($F_{(2,2)} = 1.548$, $P = 0.392$), nor in the interaction of treatment and colony ($F_{(1,145)} = 1.528$, $P = 0.220$). In the starvation assay, treatment solution did not significantly impact bumblebee survival time (Fig. 3c., $\chi_{2.88}^2 = 29.8$, $P = 0.210$) (Appendix S6).

Discussion

We demonstrate that naturally produced nectar toxins from *R. ponticum* present a previously unacknowledged threat to a solitary bee species and the native honeybee, but not to a common bumblebee species. Thus the impact of an invasive plant with toxins in its nectar on native pollinators is unequal and favours particular species depending on their tolerance for the toxin. Our results are in congruence with previous work demonstrating varying effects of secondary metabolites from invasive plants on native foliar herbivores (Shapiro 2002; Graves & Shapiro 2003; Keeler & Chew 2008).

Impacts on survival and sublethal effects

Our assays demonstrate that GTX I, but not GTX III, is the toxic component of *R. ponticum* nectar for honeybees. While GTX I consumption did not impact the survival of *A. carantonica* and *B. terrestris*, *A. mellifera* individuals in our assays died within six hours of consumption of nectar-realistic doses of GTX I, and *A. carantonica* exhibited malaise behaviours. Species-specific lethality of plant secondary compounds can result when organisms vary in their post-ingestive capacities for coping with these compounds (Berenbaum 1981; Ivie *et al.* 1983; Slansky 1992). In mammals, GTXs act on the sodium channels of cell membranes in the central nervous system, binding to the channels in their open state and preventing inactivation (Koca & Koca 2007). . Although a cursory examination of sodium channel genes and proteins in *Apis* and *Bombus* reveals that many (>60%) are similar , differences do exist (E.J. Tiedeken, J.C. Stout & James Murray, unpublished findings). These differences between bee species could suggest a mechanism for the observed differences in GTX I tolerance . Differences in metabolism of toxins or in detoxification genes could also lead to differential toxicity (Slansky 1992). *A. mellifera*'s genome contains only 46 genes coding for cytochrome P450 monooxygenases (a superfamily of enzymes associated with detoxification), constituting a reduction of > 50% compared to Dipteran species (Claudianos *et al.* 2006). Although this

indicatesa poor general capacity for detoxification, *B. terrestris* has a similar paucity of detoxification genes, and in fact has even fewer cytochrome P450 genes than *A. mellifera* (Sadd et al. 2015). It is therefore unlikely that differences in detoxification genes between these two bee species can explain the observed difference in GTX I toxicity .

Responses of pollinators to nectar secondary compounds in co-evolved native plants are wide ranging, and include increased attraction, deterrence or even death (Detzel & Wink 1993). Although nectar secondary compounds occasionally cause rapid mortality in honeybees, (reviewed in Adler 2000), empirical evidence for toxic nectar such as we present here is rare. Even sublethal impacts, such as reduced mobility and vigour (Hurst, Stevenson & Wright 2014), are often only observed when concentrations of toxins are greater than those found in nectar (Cook *et al.* 2013; Manson *et al.* 2013). If toxins are detected and avoided by pollinators (Wright *et al.* 2010), this could lead to lower fitness for plants (Adler & Irwin 2012) andthus pollinators may select for concentrations below their thresholds of impact or detection (Wright *et al.* 2013; Tiedeken *et al.* 2014). When an invasive plant species presents toxic nectar, however, native flower visitors that did not co-evolve with it could be susceptible to its secondary chemistry (Callaway & Ridenour 2004). Such a mechanism may explain the detrimental impacts we observed for *A. mellifera* and *A. carantonica* after consuming nectar GTXs from invasive *R. ponticum.* Remarkably, honeybee subspecies in the eastern part of *R. ponticum*'s native range (*Apis mellifera caucasica* and *anatolica*) readily forage on the plant. As a result they produce "mad honey" containing GTXs that cause life-threatening symptoms in humans (Silici *et al.* 2008).

Previous studies suggest that the presence of nectar secondary compounds may be an adaptive trait that helps select for the most efficient pollinators (Baker & Baker 1975; reviewed in Adler 2000). . It is possible that GTX I is acting as a toxin to screen out inefficient floral visitors, i.e. honeybee and solitary bees, in order to preserve the nectar for the best pollinators of the

plant, the bumblebees (Stout *et al.* 2006). This may be occurring via rapid co-evolution in the invasive range of *R. ponticum;* a similar adaptive response has been demonstrated with invasive plants and foliar herbivores (Keeler & Chew 2008). Alternatively the interaction between *Bombus* species and *R. ponticum* may occur via associative learning by generalist foragers.

Additional stressors

GTX consumption had no negative synergistic effects when combined with other stressors. In contrast to previous research using an alkaloid found in floral nectar (gelsemine), GTX also had no positive impacts on bumblebees challenged by pathogens (Manson, Otterstatter & Thomson 2010). *B. terrestris* may not require additional energy to cope with GTX consumption, especially if the passive defence mechanism of target-site insensitivity occurs (Slansky 1992). The lack of impact on the parasites may also be due to target site insensitivity of GTXs at the sodium channels of *C. bombi*. These assays indicate that even in the presence of additional stressors, *R. ponticum* nectar can provide a useful forage resource for *B. terrestris*.

Impacts of invasive plants on pollinators

Nectar secondary compounds in invasive plants may affect both the direction and magnitude of the impacts of invasion for pollinators. Similar results have been demonstrated previously for foliar herbivores. Invasive plants can be beneficial to herbivores that can incorporate them into their diets; if the native host has similar chemistry, herbivores may be pre-adapted and able to feed on the invasive plant (Shapiro 2002), ultimately increasing range or flight season for some native herbivores (Sims 1980; Graves & Shapiro 2003). Alternatively, invasive plants may be detrimental if larval offspring are unable to develop on the plant, or if they cannot cope with its secondary chemistry (Graves & Shapiro 2003; Keeler & Chew 2008).

Honeybees and solitary bees unable to tolerate nectar GTXs will be negatively impacted by *R. ponticum* invasion, although perhaps to different degrees. Honeybees are not seen foraging on *R. ponticum* in its introduced range (Stout *et al.* 2006; Stout 2007a), presumably because they

do not recruit nest-mates due to its toxic effects. Their complex communication is therefore more likely to prevent direct honeybee mortality from *R. ponticum* nectar consumption (Afik, Dag & Shafir 2008; Tan *et al.* 2012). In contrast, independently foraging solitary bees may be more vulnerable. Even if honeybees and susceptible solitary bees readily learn to avoid toxic *R. ponticum* nectar, by replacing native vegetation (Cross 1975; Stout & Casey 2014) and not providing a palatable alternative nectar resource, *R. ponticum* reduces the amount of food available for these bee species. Loss of floral resources is a primary driver of bee declines (Goulson *et al.* 2015), and our study demonstrates that plant invasion can decrease food availability for native bees unable to tolerate nectar toxins.

However, *R. ponticum* could provide an important flower resource for *B. terrestris* and other non-susceptible *Bombus* species, especially when they are establishing colonies in the spring. Indeed, *B. lucorum* and *B. pascuorum* colonies occur at higher density in sites invaded with *R. ponticum* when compared to uninvaded control sites (Dietzsch 2009). Invasive flowering plants may therefore increase the carrying capacity of a site for pollinators, but only if pollinator species are able to utilize the novel forage (Graves & Shapiro 2003; Tepedino, Bradley & Griswold 2008).

Conclusion

The direct impacts of invasive plant species and nectar secondary compounds on pollinators remain largely unexplored. Our study is the first to address these topics simultaneously, and to demonstrate that the latter may have considerable implications for the former. Due to the diversity of pollinator biology and physiology, drivers of pollinator decline, including invasion by alien species, can differentially impact pollinators and the ecosystem service they provide. Future studies should consider species-specific impacts in order to best conserve vital pollinator populations.

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Data Accessibility Statement

The data sets supporting this article have been uploaded as part of the Supporting Information (Appendix S7).

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Supporting Information

Additional supporting information may be found in the online version of this article

Appendix S1: Chemical analysis of *R. ponticum* nectar

Appendix S2: Oral toxicity methodology

Appendix S3: Bumblebee harnessed assay methodology

Appendix S4: Bumblebee behaviours

Appendix S5: Parasite assay methodology

Appendix S6: Survival under stress assay

Appendix S7: Data sets supporting this article

Table S1: Four *Rhododendron ponticum* populations from which plant materials were collected

Table S2: Oral LC_{50} values of GTX I for honeybees (*Apis mellifera mellifera*).

Figure S1: Average sugar concentrations (molarity) of composite *Rhododendron ponticum* nectar samples

Table 1. Five treatment solutions used in bee assays. Due to differences in bee biology or availability, not all treatments were utilized in all assays.

Treatment	Treatment description	GTX concentration	Assays in which treatment was utilized
T1	*Rhododendron ponticum* nectar	0.44 μg/mg [1]	Honeybee, bumblebee restrained and unrestrained
T2	Artificial nectar control- contains no GTX, but simulates *R. ponticum* sugar content	0 μg/mg	Honeybee, bumblebee restrained and unrestrained, and solitary bee unrestrained
T3	Artificial nectar + GTX I and GTX III[2]	0.44 μg/mg	Honeybee, bumblebee restrained and unrestrained, and solitary bee unrestrained
T4	Artificial nectar + GTX I	0.44 μg/mg	Honeybee, bumblebee restrained and unrestrained
T5	Artificial nectar + GTX III	0.096 μg/mg[3]	Honeybee, bumblebee restrained and unrestrained

[1]Concentration is expressed in μg GTX per milligram fresh weight nectar

[2]GTXI and III are both found in the nectar of *R. ponticum*, so treatment 3, which contained them in their natural ratios (0.344 μg/mg of GTX I and 0.096 μg/mg of GTX III), most closely approximated *R. ponticum* nectar. Treatments 4 and 5 were used in order to determine the individual biological activity of GTX I and GTX III.

[3] The concentration of GTX III used for treatment 5 is based on the approximate ratio of GTX I vs. GTX III in *R. ponticum* nectar

Table 2. Comparison of the behaviour of bumblebees and solitary bees fed a control solution (treatment 2) or a solution containing nectar-relevant concentrations of GTXs (treatment 3). Individuals were observed continuously for 90 seconds on 11 days throughout the 30 day assay on surviving individuals. The total proportion of time bees spent on each behaviour was calculated and compared between the two treatments. "–"a behaviour was not performed by the bee species. Bold text and ** indicates significance at $\alpha = 0.01$ and *** $\alpha = 0.001$.

Behaviour	Bumblebees		Solitary bees	
	Test statistic (W)	*P*-value	Test statistic (W)	*P*-value
Exploring	43.5	0.106	209	0.140
Still/resting	93.5	0.225	192.5	0.341
Consumption of treatment solution	61.5	0.561	181.5	0.271
Pollen manipulation	66.0	0.359	-	-
Grooming	75.5	0.862	140.5	0.458
Flying	71.0	0.977	228	**0.006** **
Distress behaviours	-	-	81	**< 0.001** ***

Figure and Table Captions

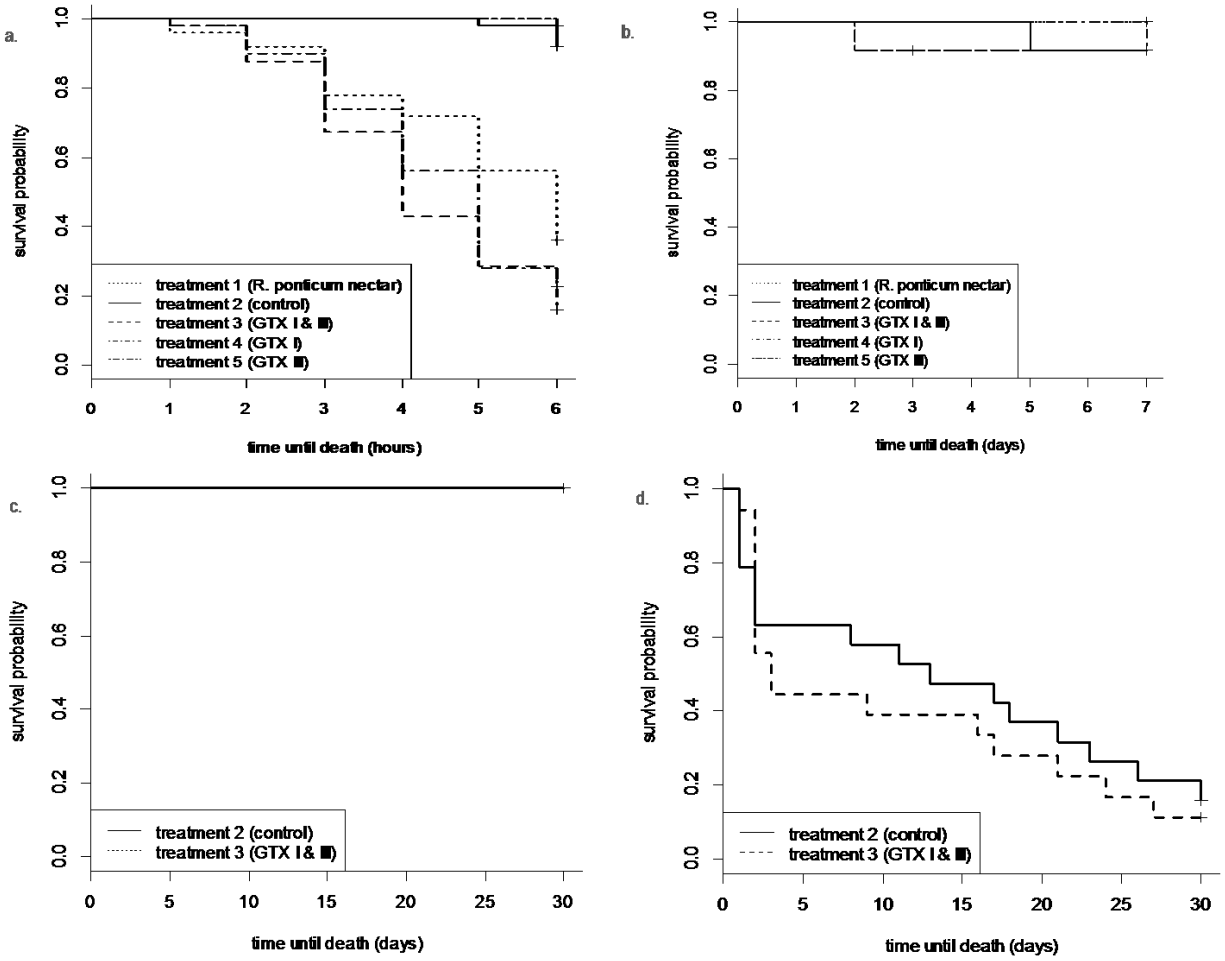

Figure 1. Survival curves of bees fed GTX from *Rhododendron ponticum.* a. honeybees fed treatments 1-5 and observed for six hours, b. bumblebees fed treatments 1-5 and observed for 7 days, c. bumblebees fed treatments 2 and 3 and observed for 30 days, and d. solitary bees fed treatments 2 and 3 and observed for 30 days. In each graph, the solid line represents the control treatment, which contained no GTXs.

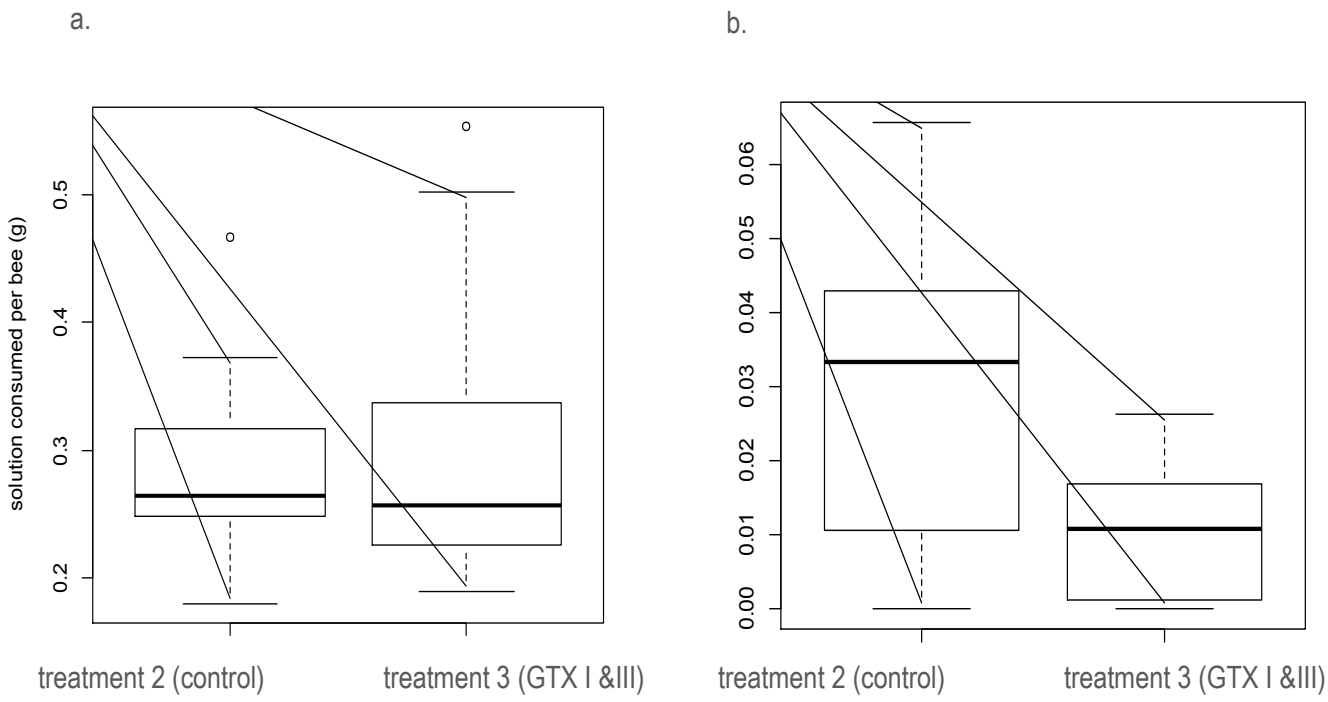

Figure 2. Comparison of consumption data for bees. a. bumblebees (n = 12) and b. solitary bees (n= 18) fed a control solution (treatment solution 2) or a solution containing nectar-relevant concentrations of GTXs (treatment solution 3) for 30 days. Consumption was measured daily in grams and controlled for evaporation. The average amount of solution consumed by each bee throughout its lifespan was compared.

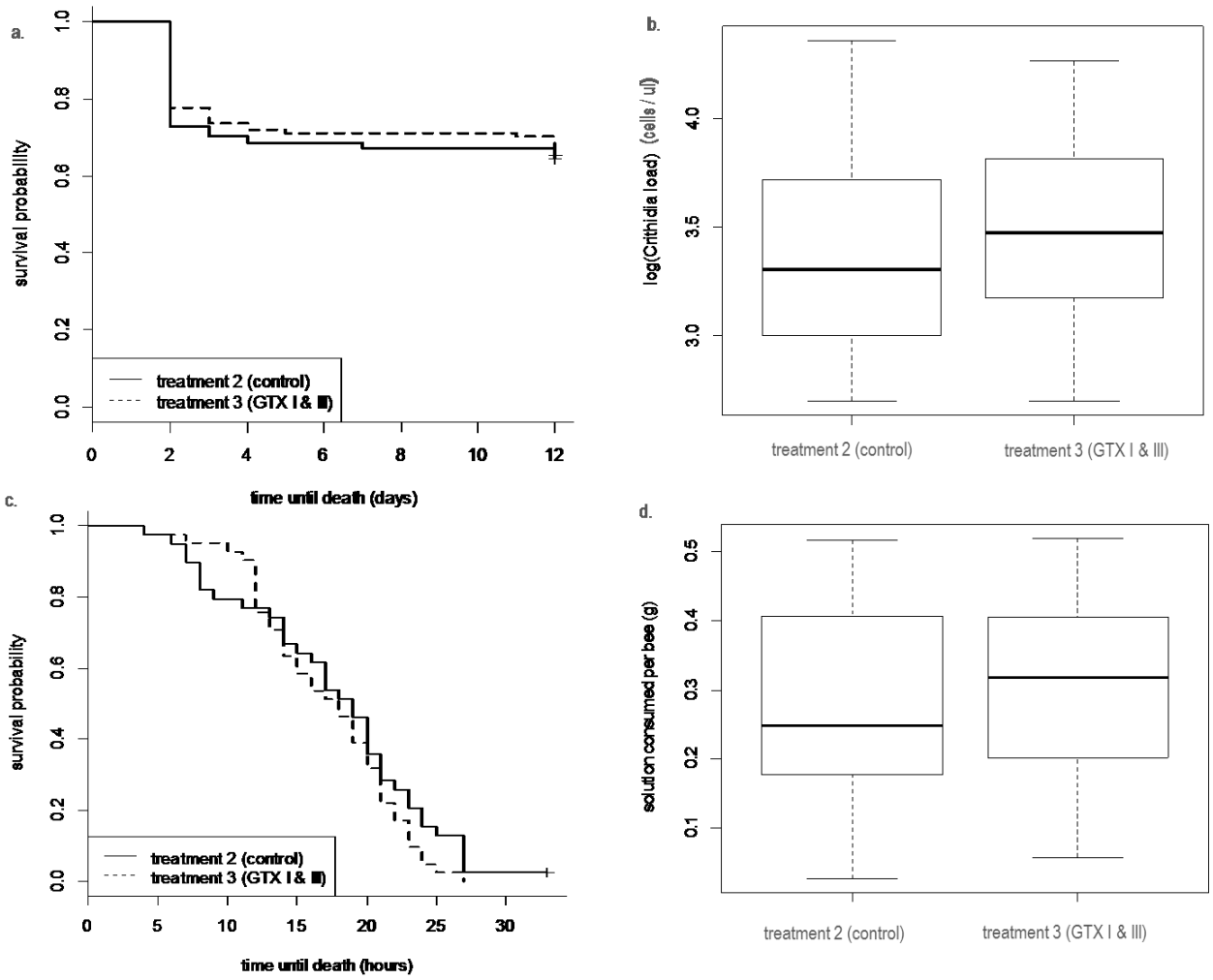

Figure 3. Combined effects of GTX consumption and additional stressors on bumblebees. a. survival and b. log (mean peak parasite load) (cells/µl) of *Bombus terrestris* workers infected with *Crithidia bombi* and fed either treatment 2 (control) or 3 (GTX I & III) for 12 days. c. survival and d. mean 24 h consumption (g) of bees fed treatment 2 or 3 for 24 h and then starved until death.