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Ecological impacts of invasive plants on pollinators: studies on an individual, species and community scale

A thesis submitted for the degree of Doctor of Philosophy

2015

Erin Jo Tiedeken

Department of Botany
School of Natural Sciences
Trinity College Dublin
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and is entirely my own work except where duly indicated and clearly acknowledged in the text.

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Summary

Pollination is a critical ecosystem service yet pollinators are experiencing worldwide declines. Although invasive alien species are cited as a major driver of pollinator declines, little is known about how pollinators are affected by plant invasion. My thesis used invasive *Rhododendron ponticum* L. as a model system to better understand how invasive alien plant species impact native pollinators on a variety of scales, from individuals, to species, to entire pollinator communities. Furthermore, the nectar of invasive *R. ponticum* contains grayanotoxins, plant secondary compounds usually associated with defence against herbivores. This paradoxical floral trait allowed me to simultaneously investigate how novel nectar secondary compounds from an invasive plant affect the health and well being of native, non-adapted pollinators, and to consider how plant traits modulate the impacts of invasion for pollinators.

Nectar secondary compounds may render the rewards provided by invasive plants unsuitable for pollinators, which could lead to negative consequences for plant reproduction via decreased pollinator visitation. However, these consequences will only become a reality if pollinators detect and are deterred by nectar secondary compounds. In a first study, I addressed this issue using a paired-choice assay to investigate whether *Bombus terrestris*, a common pollinator of invasive *R. ponticum*, was deterred from feeding on ecologically-relevant concentrations of grayanotoxins. I then broadened this study by investigating four additional secondary compounds (and two synthetic ones, see Appendix A) found in floral nectar. The deterrence threshold (the concentration at which bumblebees significantly preferred a sucrose control solution over a sucrose solution containing a compound) was always higher than the concentrations of the compounds found in nectar. Similar to other generalist bees, individuals of this bumblebee species appear to have poor acuity for the detection of nectar secondary compounds. My study suggests that generalist bees will not learn to associate floral traits with the presence of nectar secondary compounds, allowing this trait to be maintained in plant populations.

In a second study, I used a species-level approach to investigate how nectar grayanotoxins modulate the impacts of *R. ponticum* invasion for native pollinators. I also investigated the combined impacts of parasite infection and habitat loss with grayanotoxin consumption. I found that nectar-relevant concentrations of grayanotoxins are acutely toxic for the honeybee, *Apis mellifera* and a solitary mining bee species, *Andrena carantonica*, but not for *B. terrestris*, even when combined with additional stressors. Grayanotoxins therefore render the nectar of
R. ponticum unsuitable as a forage resource for two native bees, however R. ponticum may present a valuable early-spring resource for tolerant bumblebee species. I demonstrated that specific plant traits, such as nectar secondary compounds, can modulate the impacts of plant invasion for native pollinators. In the absence of tolerant pollinators, nectar secondary compounds could act as a hidden barrier to invasion for entomophilous plants.

Many invasive plant species have a relatively short flowering period, during which they provide a temporary pulse of forage resources for pollinators. This seasonal variation in floral resources may affect insect-flower interaction network structure, but few studies consider temporal variation in invaded networks. In an observational field study, I used a community-level approach to investigate the variation in floral resources, flower-visiting insect communities and interaction networks during and after the flowering of invasive R. ponticum in four invaded woodland sites. Regardless of the presence of nectar grayanotoxins, R. ponticum was highly connected to generalist flower-visitors in my networks. Although I observed compositional changes to plant and insect communities after R. ponticum flowering ceased, network structure was robust to changes in floral resources. I concluded that network structure can withstand seasonal changes in floral abundance, as long as sufficient floral resources remain to support the flower-visiting community.

In a final study I used a comparative biogeographical approach to better understand the impacts of alien plant invasion on plant-pollinator communities. Using R. ponticum as a model species, I present a novel comparison of field-replicated, quantitative insect-flower interaction networks in the native and introduced range of an invasive plant species. Overall, I found that network structure was similar for communities in both ranges, and that R. ponticum acted as a "super generalist" in native and introduced communities. I concluded that invasive plants may alter network structure in invaded communities, not just because they are novel introductions to the community, but because of the attractive and generalist nature of their floral displays.

Collectively, my work highlights that the directionality of the impact of invasive plants for pollinators, and therefore their role in pollinator decline, may vary between taxa and can be modulated by plant, pollinator and community characteristics. Future studies on pollinator decline should be carried out at multiple scales and should consider multiple interacting global change pressures.
To my family, both near and far
Acknowledgements

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the solitary bee assays for Chapter 3. Thanks to Sharon especially for always being there for a chat and a cup of tea when I needed it.

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## List of main contributors to each chapter

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Chapter 1

General Introduction
1 General Introduction

1.1 Importance of pollinators

1.1.1 Pollination: An ecosystem service

Ecosystem services are the economic benefits provided to people by nature (Millennium Ecosystem Assessment 2005), and one service of key importance is pollination (Kearns et al. 1998, Klein et al. 2007). Pollination occurs when pollen is transferred from the anthers (male parts) to the stigma (female parts) of a flower of the same plant species, resulting in fertilization. While pollen transfer can be achieved through abiotic means (via water or wind), the majority of flowering plant species benefit from biotic pollination (Klein et al. 2007, Ollerton et al. 2011), whereby pollen is transported on the body of an animal pollinator.

1.1.2 Value of pollinators

Animal pollination is important both ecologically, for the maintenance of biodiversity and ecosystem functioning, and economically, for the production of many agricultural crops (Ollerton et al. 2011, Calderone 2012). It is estimated that 87.5% of the world's angiosperm species benefit from animal pollination (Ollerton et al. 2011), and over 60% of tested species exhibit pollen limitation (Burd 1994, Ashman et al. 2004, Knight et al. 2005b). Economically, pollination services were valued at €153 billion per annum worldwide in 2005 (Gallai et al. 2009), but production of pollinator-dependent crops is increasing disproportionally to pollinator-independent crops (Aizen et al. 2008a), so this value is likely to increase. Klein et al. (2007) found that 87 of the 124 main crops used directly for human consumption are dependent on pollinators to some extent. Although humans depend on a few pollinator-independent staple crops for the majority of our caloric intake, animal-pollinated crops still represent over one third of the world's overall food supply (Klein et al. 2007), and the diversity of nutrients in our diets would be greatly diminished without them (Eilers et al. 2011). Animal pollination therefore plays a significant role in food security (Cock et al. 2012, Garratt et al. 2014), especially in light of increasing demands for pollination services (Breeze et al. 2011). Finally, pollinators also have great cultural significance. Managed honeybees in particular are valued not only for pollination services (Morse and Calderone 2000), but also for the hive products that result from beekeeping, a past-time practiced by humans for thousands of years (Michener 1974, Potts et al. 2010b). Fortunately, public awareness of important wild bee species is also increasing (Plate 1, Breeze et al. 2011, Garibaldi et al. 2013, Rogers et al. 2014).
Loss of Bees Can Affect Plants' Ability to Reproduce, Study Finds


1.2 Pollinator decline

1.2.1 Evidence for pollinator decline

Due to dramatic, wide-ranging declines in both domestic and wild pollinators, the world is thought to be undergoing a “pollination crisis” (Allen-Wardell et al. 1998, Kearns et al. 1998, but see Ghazoul 2005). The first empirical evidence for pollinator decline came from regional reports on managed honeybees (*Apis mellifera*) (Southwick and Southwick 1992, Morse and Calderone 2000). In the USA, domestic honeybee colonies decreased by 59% from 1947 to 2005 (Natural Research Council 2007) while in Europe, a 25% decrease in colonies was observed between 1985 and 2005 (Potts et al. 2010b). Studies subsequently documented declines in variety of wild pollinator taxa; for example, 71% of British butterfly species have declined since the 1980’s (Thomas et al. 2004), and bumblebee richness and abundance has decreased in Europe (Biesmeijer et al. 2006, reviewed in Goulson et al. 2008) and the USA (Colla and Packer 2008, Grixti et al. 2009, Cameron et al. 2011) in the past several decades. While direct evidence for declines in additional wild pollinator taxa are largely unavailable due to the scarcity of data for these species (reviewed in Potts et al. 2010a), studies suggest that anthropogenic changes to the landscape greatly reduce overall pollinator richness and abundance (Winfree et al. 2009, Montero-Castaño and Vila 2012). Furthermore, declines appear to be biased towards dietary or habitat specialist species (Biesmeijer et al. 2006,
Goulson et al. 2008, Potts et al. 2010a). Parallel declines in insect pollinated plants have also been recorded (Biesmeijer et al. 2006), although a causal link and the directionality of the relationship with pollinator declines remains difficult to demonstrate. The number of studies investigating the drivers of pollinator decline has increased dramatically in the past two decades (Figure 1.1), as researchers struggle to understand and conserve pollinator populations.

![Figure 1.1](image.png)  
Figure 1.1 The annual number of publications on pollinator and/or bee decline has increased dramatically in the past decade. Search conducted in ISI Web of Science's Core Collection using the time period from 1950-July 2014, using the following search terms: Title = (pollinator* OR bee OR bees OR pollinat*) AND Topic = (declin*).

### 1.2.2 Drivers of pollinator decline

Anthropogenic alterations to the environment that result in decreased floral resources are likely to be primary drivers of pollinator decline, particularly for obligate flower-visitors such as bees (Goulson et al. 2008, Potts et al. 2010a). Specifically, the following five drivers are thought to be the main contributors to pollinator declines: habitat disturbances (including habitat loss, fragmentation and landscape alteration, Kremen et al. 2002, Steffan-Dewenter et al. 2002, Ricketts et al. 2008, Winfree et al. 2009), increased exposure to agrochemicals (Cresswell 2011, Goulson 2013), the spread of pathogens and parasites (Cox-Foster et al. 2007, Evison et al. 2012, Fürst et al. 2014), climate change (Roberts et al. 2011, Bartomeus et al. 2013), and the introduction of alien species (Stout and Morales 2009, Williams et al. 2011, Montero-Castaño and Vila 2012). Furthermore, bees are exposed to multiple drivers...
simultaneously that can interact and have non-additive effects (Gonzalez-Varo et al. 2013, Vanbergen and Insect Pollinators Initiative 2013). Although most research investigates these five main drivers in isolation (but see Bartomeus et al. 2010, Schweiger et al. 2010, Williams et al. 2011, Baron et al. 2014), investigating the impacts of multiple interacting pressures on pollinators is considered a priority (reviewed in Gonzalez-Varo et al. 2013).

1.3 Plant-pollinator ecology

The relationship between plants and pollinators is one of the best-studied mutualisms in ecology (Proctor et al. 1996, Waser et al. 1996, Kearns et al. 1998). Even though pollination fits the definition of a mutualistic relationship, it is truly a balanced mutual exploitation (Inouye 1980, Howe 1984, Dietzsch 2009); the pollinator is in search of food for itself or its brood in the form of carbohydrate-rich nectar or protein-rich pollen, while the plant requires the transfer of pollen to achieve reproductive success. Limiting the removal of floral resources and increasing pollen transfer benefits the plant, while maximizing foraging efficiency benefits the pollinator (Kearns et al. 1998, Goulson 2010). The resulting conflict has led to coevolution between plants and pollinators, each reciprocating on an evolutionary time scale to the changes in its partners' traits (Proctor et al. 1996). This section first discusses some aspects of the biology, life history and diversity of pollinators that must be understood in order to conserve these vital populations, and second reviews the nature of plant-pollinator communities.

1.3.1 Characteristics of pollinators

The majority of the world's estimated 300,000 flower-visiting species (> 70%) are insects (Nabhan and Buchmann 1997), so I will focus only on this pollinator group. Due to 1.) their efficiency as pollinators of crop flowers, (Delaplane et al. 2000), 2.) their commercial availability and 3.) the relative ease of handling them in the laboratory (Baer 2003, Keller and Jemielity 2006), bees (superfamily Apoidea) represent the best studied flower-visiting insect taxa (Michener 1974). Even though wild bees are often the most effective pollinators (Garibaldi et al. 2013, Rogers et al. 2014), much literature focuses on the managed honeybee, *Apis mellifera* (Ollerton et al. 2012a). This bias has resulted in large knowledge gaps in regards to the biology and life history of many wild bee species.

In comparison to the plants they rely on, pollinators are highly mobile, often travelling hundreds of meters in search of food resources (Knight et al. 2005a). Some insect pollinators are facultative flower visitors (they consume floral resources but do not depend on them), but
many are obligate (they require floral resources for at least part of their life cycle, see Plate 2 for examples) (Junker and Blüthgen 2010). Obligate pollinators may feed on nectar (as a source of carbohydrates (Nicolson 2011)) and pollen (as a source of protein (Michener 1974)) both as adults and larvae, e.g. bees, some wasp and moth species, or alternatively only as adults, e.g. Syrphids and most wasps (Proctor et al. 1996). Pollinators utilize a variety of cues in order to identify flowers with the best quality rewards, including, but not limited, to floral colour, scent, age, size, symmetry, abundance and sex (Proctor et al. 1996, Goulson 2010). Pollinating species with advanced social structures also communicate reward quality to nest mates (Dornhaus and Chittka 2001, Afik et al. 2008), and many bee species leave repellent scent marks on flowers that indicate a decrease in reward quality to both conspecific and heterospecific species (Stout et al. 1998, Stout and Goulson 2001, Goulson 2010). Pollinators also vary greatly in life history and biology, ranging from solitary species to advanced eusociality (Michener 1974), and from oligolectic to polyleptic (Waser and Ollerton 2006). These differences should be considered from a conservation perspective because they may result in taxon or species-specific responses to drivers of decline.

Plate 2. Examples of obligate and facultative flower-visiting (and potential pollinating) insects. From top left to bottom right: Vespa spp. (facultative), Pieris spp. (obligate), Cerambycidae spp. (facultative), Bombus spp (obligate), Diptera spp. (facultative), and Syrphid spp. (obligate). (Photo credit: Paul A. Egan).
1.3.2 Plant-pollinator communities

Even though plant-pollinator mutualisms are often associated with extreme specialization and strong co-evolutionary relationships (e.g. yuccas and figs, Pellmyr and Thompson 1992, Patel and McKey 1998), generalization is the rule rather than the exception in these communities (Waser et al. 1996, Kearns et al. 1998, Memmott 1999, Waser and Ollerton 2006). Plants benefit from generalization because of the spatial and temporal variability exhibited by highly mobile pollinators. Pollinators, likewise, benefit from generalization because of the cost of travel to forage resources, the similarity of floral rewards and the long lifespan of pollinators relative to the flowering period of many plants (Waser et al. 1996).

The generalist nature of plant-pollinator communities stresses the need for a community-level approach to the study of these systems (Memmott 1999, Lopezaraiza-Mikel et al. 2007, Vázquez et al. 2009). One method that addresses this need is the construction of insect-flower (or plant-pollinator) interaction networks. These networks consist of interactions between flower-visitors and the plants they visit, and can be qualitative (demonstrating only whether or not a link exists between partners), or quantitative (demonstrating the relative strength of each link) (Dornann et al. 2009). Insect-flower interaction networks can help us better understand community structure, species interactions, (Vázquez et al. 2009) and can also answer conservation questions (Lopezaraiza-Mikel et al. 2007, Tylianakis et al. 2007, Memmott 2009, Kaiser-Bunbury et al. 2011). Because network structure is vulnerable to the identity, diversity and behaviour of species, metrics that describe network structure may show changes in response to perturbations before a loss of species diversity is obvious (Tylianakis 2008).

Most insect-flower interaction networks tend to exhibit several consistent patterns. For example, network connectance, or the percentage of all possible interactions in a network that are actually established, is usually low (Jordano 1987, Olesen et al. 2002). In addition, the number of insects usually outweighs the number of plants in a ratio of approximately 4:1 (Blüthgen et al. 2007). Insect-flower interaction networks also tend to be nested, meaning there is a tendency of specialist species to interact with a subset of interaction partners of highly connected species (Bascompte et al. 2003, but see Dornann et al. 2009). This nested structure generates networks that are asymmetric, both in degree and strength (Bascompte et al. 2003, Vázquez and Aizen 2004, Bascompte et al. 2006); that is, if a plant depends strongly on a pollinator, the pollinator depends weakly on the plant, and vice versa. These two properties, nestedness and asymmetry, are thought to confer stability and robustness to these
networks, especially in the face of species extinctions (Memmott et al. 2004). However, recent models suggest that drivers causing pollinator decline will induce pollinator populations to collapse once a tipping point is reached, and that recovery from this collapse will be challenging (Lever et al. 2014). Research is also revealing that network structure can be temporally and spatially variable (Alarcón et al. 2008, Olesen et al. 2008, Petanidou et al. 2008, Dupont et al. 2009), although few studies take such variability into account.

As software for creating and analyzing insect-flower interaction networks became readily available (e.g. Dormann et al. 2008), the field saw a veritable boom in network analyses. Unfortunately, the sheer volume of studies has lead to 1.) inconsistencies in sampling methods and 2.) confusion over the terminology used to describe these networks. Data for network analysis can be collected via two methods: transect walks, where all insect-flower interactions that occur in a transect of a fixed length are recorded as the observer walks at a steady speed, or timed observations, where all insect-flower interactions that occur during a fixed time period are recorded as the observer watches a plant species or quadrat (Gibson et al. 2011). Any sampling method will provide only a snapshot of the network that will inevitably vary from the underlying "true" network structure (Vázquez et al. 2009). Still, differences in the allocation of sampling effort between these two main methods can significantly alter some network parameters and should therefore be carefully considered (Gibson et al. 2011). Furthermore, terminology in this field can be misleading; networks are often referred to as "plant-pollinator networks," when they do not measure the pollination efficiency of visitors (Memmott et al. 2004, Vázquez et al. 2009, Gibson et al. 2011). Visitation can be a poor proxy for pollen deposition (King et al. 2013), therefore networks constructed from visitation data should be referred to as "insect-flower interaction networks" (but see Vázquez et al. 2005, and Chapter 9, Waser and Ollerton 2006).

The variable biology and life history of pollinators, combined with their highly mobile nature and tendency for generalist interactions, demands that they be studied at a variety of scales. Individual or species-level lab-based assays are necessary for understanding the physiology and life cycles of pollinators, while observational and experimental field studies at the community level provide insights into the structure of networks and responses to perturbations. In order to best conserve pollinator populations, a combination of these studies should be used to understand the mechanisms behind the drivers of pollinator decline.
1.4 Invasive alien species

As humans continue to develop worldwide pathways of trade and travel, we inevitably introduce species to areas beyond their natural or historic ranges (Davis 2009). Introduced species that become invasive can impact all aspects of biodiversity, including pollinators and pollination services (Stout and Morales 2009, Potts et al. 2010a). Although the impacts of invasive pollinators and pathogens are comparatively well studied (Bach 1991, Goulson et al. 2008, Meeus et al. 2011), relatively little work has considered the impacts of invasive alien plants on native pollinators.

1.4.1 Introduction to invasive alien species, with a focus on plants

A species is considered introduced (also alien, non-indigenous, non-native, or exotic) when it is transported either accidentally or intentionally by humans beyond natural dispersal barriers (Richardson et al. 2000b, Colautti and MacIsaac 2004). The tens rule predicts that only 10% of species will reach each subsequent stage in the invasion process; that is, 10% of introduced species become casual (i.e. they do not form self-replicating populations but persist due to repeated introductions), 10% of casual species become naturalized (i.e. they survive and sustain self-replacing populations for several generations), and 10% of naturalized species become invasive (i.e. they rapidly spread considerable distances from introduction sites and reproduce, often in large numbers or at high densities) (Richardson et al. 2000b, Richardson et al. 2011). Even though only a small proportion of introduced species actually become invasive (Richardson et al. 2011), biological invasions are pervasive drivers of global environmental change and can impact biodiversity, natural resources and ecosystem services (Millennium Ecosystem Assessment 2005, Simberloff et al. 2013). Economically, invasive alien species are estimated to cost the United States and Europe over $120 billion and $10 billion per year respectively (Pimentel et al. 2005, DAISE 2009), and even these values likely underestimate the true costs (Born et al. 2005).

Invasive alien plants have infiltrated every ecosystem on earth, and the incidence of plant invasion has continuously increased since the eighteenth century (Pyšek et al. 2003). Much work has investigated how invasive plants impact the growth and reproductive success of native plants, and overall negative impacts have been found to dominate these relationships. Plant invasion can therefore change native plant community structure or composition (Cross 1982, Pyšek and Pyšek 1995, Colak et al. 1998) and ecosystem processes (Levine et al. 2003, but see Rodriguez 2006 for a review of facilitative effects of invasive spp.). The mechanisms
for these impacts may be abiotic (e.g. changes in hydrology, nutrient cycling, or soil pH) or biotic (e.g. direct competition with natives for light and soil moisture, or disruption of interactions between natives and their mutualistic partners) (reviewed in Richardson et al. 2000a, Levine et al. 2003, Stout 2011). The density and abundance of invasive alien plants influences the severity and direction of impacts for native plant communities (Parker et al. 1999, Munoz et al. 2008, Dietzsch et al. 2011). Invasive plants have the potential to impact pollinators indirectly via such effects on native plant communities (Stout and Morales 2009).

In addition to investigating the impacts of invasion, a major goal of invasion biology is to understand what factors make an invasive species successful, and what traits contribute to habitat resistance or susceptibility to invasion (Richardson and Pyšek 2006, Simberloff 2011). For example, the enemy release hypothesis states that invasive plants are successful in introduced ranges because they experience decreased pressure from natural enemies that occur in their native range, resulting in increased allocation of resources to competition or reproduction (Darwin 1859, Elton 1958, Keane and Crawley 2002, Colautti et al. 2004). Traits specific to invasive alien species may also increase invasability (Milbau and Stout 2008); for example the novel weapons hypothesis states that alien species that contain biochemicals will have a competitive advantage because the novel biochemicals are more effective in antagonistic biotic interactions (e.g. allelopathy) (Callaway and Ridenour 2004). Growth form, clonality, time of flowering and breeding system have also been suggested as important plant traits that may contribute to invasiveness, although finding consistent patterns across taxa has proven difficult (reviewed in Pyšek and Richardson 2007, Milbau and Stout 2008, Higgins and Richardson 2014).

More recently, researchers have considered the role that pollinators and other mutualistic interactions (e.g. seed dispersal or symbioses between plant roots and microbiota) play in the invasion process (Richardson et al. 2000a, Mitchell et al. 2006). The mutualist facilitation hypothesis says that replacement of lost mutualisms from a plants’ native range with new mutualist partners in the exotic range is key to the establishment and spread of invasive alien species (Richardson et al. 2000a). Theory suggests that self-compatible plants that reproduce via asexual propagation and self-pollination will be better invaders, because they will be less prone to pollinator limitation in the exotic range (Baker 1965). Nevertheless, pollination does not often provide an effective barrier to invasion (Richardson et al. 2000a, Rodger et al. 2010); many introduced plant species require or benefit from visitation and pollen transfer by animals
1.4.2 Impacts of plant invasion on native plant pollination

The majority of studies investigating the impacts of plant invasion on plant-pollinator mutualisms focus on native plant pollination. Invasive alien plants are frequently introduced to non-native environments for their ornamental value (Reichard and White 2001). They therefore tend to have large, attractive flowers that are often heavily scented and produce copious nectar and pollen (Bjerknes et al. 2007, Stout and Morales 2009); invasive alien plants are also often highly abundant. These characteristics can make invasive plants attractive to generalist pollinators. On the one hand, invasive plants may compete with native plants for pollination services (Chittka and Schürkens 2001, Morales and Traveset 2009), but conversely they may facilitate pollination to native species (Moragues and Traveset 2005, Ghazoul 2006, Lopezaraiza–Mikel et al. 2007). They may also have no effect on native plant pollination or reproduction (Totland et al. 2006, Dietesch et al. 2011). Although results can be variable, meta-analyses reveal a predominantly negative impact of invasive plants on the pollination and reproduction of native plants that increases with phenotypic matching and phylogenetic relatedness (Bjerknes et al. 2007, Morales and Traveset 2009). Although these studies provide information on how plant invasion impacts pollinator foraging behaviour, they do not consider the direct impacts of plant invasion for pollinators.

1.4.3 Impacts of plant invasions on plant-pollinator networks

Recently, studies have moved beyond pair wise comparisons of how plant invasions impact native plant pollination, and have instead started to consider impacts for entire plant-pollinator communities (Memmott and Waser 2002, Lopezaraiza–Mikel et al. 2007, Bartomeus et al. 2008, Bartomeus et al. 2010, Kaiser-Bunbury et al. 2010). In order to reproduce and spread, entomophilous introduced plants must integrate into plant-pollinator networks (Chittka and Schürkens 2001, Stout 2007b, Russo et al. 2014); invasive plants therefore have the potential to alter native plant-pollinator network structure. So far, empirical evidence suggests that introduced plants integrate into networks through native and alien generalist pollinators (Memmott and Waser 2002, Olesen et al. 2002, Morales and Aizen 2006), and that they often interact strongly with native community members, sometimes receiving significantly more pollinator visits than native plant species (Lopezaraiza–Mikel et al. 2007, Aizen et al. 2008b, Bartomeus et al. 2008, Padrón et al. 2009). Invasive alien plants that
encourage the addition of new, generalist pollinators can play particularly important topological roles in network structure (Albrecht et al. 2014).

Several studies have specifically investigated the impact of plant invasion on plant-pollinator network structure, but the results are somewhat conflicting. Some studies report minimal impacts, indicating network structure is largely resilient to invasion (Vilà et al. 2009). However, plant invasion has also been shown to increase the nestedness of networks (Bartomeus et al. 2008), decrease vulnerability and interaction evenness (Kaiser-Bunbury et al. 2011), and decrease connectance among native species (Aizen et al. 2008b). The way in which an invasive plant integrates into mutualistic networks (via additive or competitive mechanisms), will impact its influence on network structure (Russo et al. 2014). Furthermore, species that are incorporated as generalists, forming a high frequency of mutualistic interactions with many resident pollinator species, are likely to have a greater impact than specialist introduced species (Russo et al. 2014). Although mutualistic networks are known to vary over time and space (Alarcón et al. 2008, Petanidou et al. 2008), very little work has considered such variation in invaded mutualistic networks (but see Bartomeus et al. 2008, Kaiser-Bunbury et al. 2011).

1.4.4 Impacts of plant invasion on pollinators

Surprisingly, investigations of the direct impacts of plant invasions on pollinators are the most infrequent studies in this field (Stout and Morales 2009). To date approximately 15 studies have been carried out (see Appendix B, Table B.1). It is often assumed that plant invasion negatively impacts pollinator populations (Rodriguez 2006, Litt et al. 2014). However, observational and experimental studies have found that impacts can be positive, negative, or neutral and may depend upon factors such as pollinator taxa, habitat and the identity of the invader (Montero-Castaño and Vila 2012). Ultimately, the direction of effects will depend on how invasive alien plants impact resources essential to pollinators, for example nesting sites (Edwards and Jenner 2005) or larval or adult forage resources (Graves and Shapiro 2003, Bjerknes et al. 2007).

On the one hand, plant invasion may benefit native pollinators by providing an important novel source of pollen and nectar (Rodriguez 2006, Bjerknes et al. 2007, Stout and Morales 2009). Given their abundance and the fact that they often possess generalist attractive floral displays, invasive plants may in be ecologically equivalent to mass-flowering crops, which indeed can increase forage resources and therefore generalist pollinator densities in some
agricultural landscapes (Westphal et al. 2003). Some research does suggest that introduced plants directly benefit pollinators by providing novel forage resources, especially in areas poor in plant diversity/abundance (Baskett et al. 2011). For example, 34% of California's native butterfly species reportedly oviposited or fed on introduced plant taxa (Graves and Shapiro 2003), and native bee species in Utah (Tepedino et al. 2008) and in central California and southern New Jersey (Williams et al. 2011) utilize a variety of abundant invasive plant species as forage resources.

However, plant invasion can also negatively impact pollinators (Ernst and Cappuccino 2005, de Groot et al. 2007, Moron et al. 2009, Hanula and Horn 2011). Negative impacts may occur when floral rewards provided by the invasive species displace preferred plants and are not available or suitable for native pollinators. Specialist pollinators are more likely to be negatively impacted by plant invasions than generalist species, because they may not be able to incorporate the invasive plant into their diets (Graves and Shapiro 2003, Williams et al. 2011, Montero-Castaño and Vila 2012). In order to be useful to native pollinators, the nectar and pollen provided by invasive plants must be morphologically accessible and spatially and temporally available (Stout and Morales 2009). Even if floral rewards from invasive plants are available to native pollinators, if plant invasion severely reduces flowering species diversity, sufficient resources may not remain after the invasive species ceases flowering. Such a scenario could be detrimental for long-season foragers, like honey and bumblebees (Moron et al. 2009, Stout and Casey 2014). Finally, invasive species must also provide rewards that are of suitable nutritional value (Stout and Morales 2009). For example, Graves and Shapiro (2003) found that at least three butterflies native to California laid their eggs on introduced plant species that were toxic to larvae. The presentation of toxic or non-nutritious rewards by invasive species is an area that has received little attention, but could have serious impacts on the way pollinators respond to plant invasion.

1.5 Natural (and synthetic) chemicals in floral nectar

1.5.1 Toxic nectar: an introduction

Sugar-rich floral nectar (Lüttge 1977) is the most common reward provided by angiosperms for pollinating animals (Baker and Baker 1975, Proctor et al. 1996, Heil 2011). Floral nectar was originally thought to be comprised of simple sugars and water; however, in the 1970’s and 80’s, researchers surveying the nectar of hundreds of plant species found that up to 10% of the dry weight of most nectar contains additional compounds, including non-protein amino acids,
lipids, mineral ions, and surprisingly, plant secondary compounds (Baker 1977, Lütgete 1977). Plant secondary compounds are low molecular weight compounds that are not directly produced by or involved in primary metabolic processes such as respiration and photosynthesis (Seigler 1998). Secondary compounds have various functions within plants but they often play defensive roles against antagonists such as herbivores (when expressed in vegetative structures) and competitors (when exuded from roots) (Fraenkel 1959, Rosenthal and Berenbaum 1992, Wink 1993, Seigler 1998). Hereafter, I define "toxic nectar" as nectar that has the potential to deter or poison flower-visitors, usually, but not always, due to the presence of plant secondary compounds (Adler 2000).

Toxic nectar is a seemingly paradoxical phenomenon; why would a substance that mainly functions as a reward for pollinators contain potentially toxic or deterrent plant metabolites? Nevertheless, secondary compounds, such as alkaloids, iridoid glycosides, phenolics and terpenes, occur in the floral nectar of species in at least 21 plant families (Adler 2000). In a broad survey of hundreds of plant species, Baker (1977) found that 36% of species contained phenolics and nine percent contained alkaloids in their floral nectar. Even more species are being added to the list as technological advances continue to allow chemists to screen for previously undetectable concentrations of secondary compounds (London-Shafir et al. 2003, Manson et al. 2012, Cook et al. 2013). The frequency with which invasive plant species express toxins in their floral nectar is unknown, but it may be a common occurrence given the widespread geographic and phylogenetic incidence of toxic nectar in native species.

In addition to natural plant secondary compounds, synthetic agrochemicals can also occur in floral nectar. Systemic insecticides are water soluble compounds that are readily absorbed by plants through roots or leaves, and subsequently travel throughout the remaining tissues, providing protection to all parts of the plant (Goulson 2013). Although systemic insecticides protect against insects that are difficult to control using conventional foliar sprays (e.g. root-feeding or boring insects), they may have negative consequences for mutualistic pollinators because they also occur in nectar and pollen (EFSA 2012). The concentrations of systemic insecticides in nectar do not appear to impact the longevity of bees (Cresswell 2011), but they can have substantial sublethal effects (Gill et al. 2012, Whitehorn et al. 2012), and their impacts on non-bee pollinator taxa remain largely unexplored (Goulson 2013). Although I will hereafter focus solely on natural plant toxins in floral nectar, the methodology used and the insights gained from natural systems may aid in our understanding of the very topical issue of the impacts of agrochemicals on pollinator health (see Appendix A). Both synthetic and
natural toxins in floral nectar have the potential to influence pollinator behaviour and physiology (Elliott et al. 2008, Manson and Thomson 2009, Manson et al. 2010, Manson et al. 2013), as well as interactions between plants and flower-visitors, and could thus ultimately impact plant pollination and reproductive success (Adler and Irwin 2005, Adler and Irwin 2012).

1.5.2 Ecological significance of toxins in floral nectar

The ecological significance of toxic nectar is not fully understood, however several authors have proposed adaptive and non-adaptive hypotheses to explain the functions of secondary compounds in floral nectar (Adler 2000, Raguso 2004). Many of these hypotheses were comprehensively reviewed by Adler (2000), however several new hypotheses have been developed since then and are reviewed in Table 1.1.

The first hypothesis suggests that toxic nectar acts as a filter for flower visitors, promoting visitation from only the most efficient pollinators that are tolerant to secondary compounds (pollinatory fidelity hypothesis, Baker and Baker 1975). In this way, toxic nectar may function similarly to other floral structures that require specialization of pollinators, such as closed corollas (Rhoades and Bergdahl 1981). A related concept, the nectar robber hypothesis, says that nectar toxins may defend against floral larcenists that decrease plant fitness (Janzen 1977, Baker 1978). Both of these hypotheses assume that efficient pollinators are tolerant to toxins, while antagonist visitors are susceptible; however it is unlikely that such species-specific responses occurred immediately after this plant trait arose (Adler 2000). It is perhaps more likely that pollinators eventually specialized on toxic nectar that persisted for another reason, such as preventing the degradation of sugar-rich nectar (antimicrobial hypothesis, Hagler and Buchmann 1993). Other possible functions of toxic nectar include: altering pollinator behaviour to increase pollen transfer (drunken pollinator hypothesis, Ehlers and Olesen 1997) or to optimize the volume of nectar removed (nectar volume hypothesis, Kessler and Baldwin 2006), attracting efficient pollinators through low doses of pharmacologically active compounds (pharmacological hypothesis, Singaravelan et al. 2005), or inducing self-medication in pollinators by protecting against harmful pollinator pathogens/parasites (self-medication hypothesis, Manson et al. 2010). These hypotheses are not mutually exclusive and the function of nectar toxins may differ between taxa.
Table 1.1 Hypotheses proposed by authors to explain the occurrence of toxic or deterrent secondary compounds in floral nectar. Studies that tested or provided indirect support for hypotheses are listed: +/- indicates a study’s findings supported or did not support the hypothesis respectively.

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Definition and responsible authors</th>
<th>Studies testing hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacological</td>
<td>Secondary compounds in nectar have positive pharmacological or psychoactive effects on pollinators leading to pollinator-mediated selection for low concentrations in nectar. (Singaravelan et al. 2005)</td>
<td>(Dettzel and Wink 1993) +/- (Wright et al. 2013) - (Kessler and Baldwin 2006) + (Kessler et al. 2008) + (Tadmor-Melamed et al. 2004) +</td>
</tr>
<tr>
<td>Reduced nectar volume</td>
<td>Toxic nectar optimizes the number of flower visitors per volume of nectar produced by acting as a mild repellent. (Kessler and Baldwin 2006)</td>
<td>(Kessler and Baldwin 2006) + (Kessler et al. 2008) + (Tadmor-Melamed et al. 2004) + (Manson et al. 2010)</td>
</tr>
<tr>
<td>Self-medication</td>
<td>Pollinators select for nectar toxins that lower parasite loads. (Manson et al. 2010)</td>
<td>(Manson et al. 2010)</td>
</tr>
</tbody>
</table>
Alternatively, toxic nectar may not have an adaptive function, but instead could be a consequence of defence of other plant tissues; this explanation has been dubbed the pleiotropy hypothesis (Adler 2000). Animal-pollinated plants need to defend themselves from antagonists, such as herbivores, and simultaneously attract mutualist pollinators. Plant traits, including the production of secondary compounds, may therefore be simultaneously under selection from conflicting pressures from antagonists and mutualists (Strauss et al. 1999). Whether or not toxic nectar results in a fitness benefit or cost for plants is key towards understanding if this plant trait is adaptive. Studies of a perennial vine, *Gelsemium sempervirens*, indicate that the presence of the toxic alkaloid gelsemine in its nectar has negative impacts on pollination and male and female reproduction (Adler and Irwin 2005, Adler and Irwin 2012). In contrast, the norditerpene alkaloids in the nectar of *Delphinium barbeyi* do not negatively impact female plant reproduction (Manson et al. 2013), and evidence suggests the nectar alkaloid nicotine actually has positive impacts on the fitness of *Nicotiana attenuata* (Kessler et al. 2008). Further studies on additional plant species are needed, but again, results may vary between plant taxa.

### 1.5.3 Impacts of toxic nectar on pollinators

Extensive investigations of the impacts of plant secondary compounds on foliar herbivores have found a range of responses, including effects on dietary preferences and behaviours, physiology and longevity (Fraenkel 1959, Rosenthal and Berenbaum 1992). In comparison, the impacts of these metabolites on pollinator health and well being are poorly studied. The concentrations of secondary compounds in floral nectar are often much lower than in other plant tissues, such as leaves, flowers or fruits (Adler and Irwin 2012, Manson et al. 2012, Cook et al. 2013, Gosselin et al. 2013). Direct, negative impacts on pollinator survival are therefore rare (but see Pryce-Jones 1942, Carey et al. 1959, Crane 1977, Majak et al. 1980); instead nectar secondary compounds tend to elicit sublethal physiological or behavioural responses from pollinators. It is clear that the direction and magnitude of the effects of nectar secondary compounds on pollinators are dependent upon the secondary compound concentration, identity and the quality of the nectar reward (London-Shafir et al. 2003, Liu et al. 2007, Tan et al. 2007, Manson et al. 2013).

The majority of studies considering the impacts of toxic nectar on pollinators investigate effects on foraging behaviour and preference. The findings, however, often are conflicting; nectar toxins can be deterrent (Detzel and Wink 1993, Adler and Irwin 2005, Gegear et al. 2007, Adler and Irwin 2012), attractive (Detzel and Wink 1993, Singaravelan et al. 2005), or
have no impact on pollinator preferences (Landolt and Lenczewski 1993, Manson et al. 2012, Manson et al. 2013). Furthermore, the impacts of nectar toxins on pollinator preference can be influenced by ecological context. Apis cerana preferred honey without secondary compounds to honey containing triptolide when given a choice, but freely fed on the toxic honey when the control honey was removed (Tan et al. 2007). Similarly, Gegear et al. (2007) found that bumblebees readily fed on sucrose solutions containing the alkaloid gelsemine when it was offered in isolation, but preferred equally rewarding control solutions containing low or no gelsemine when they were offered in combination. Reward quality may also impact pollinator foraging decisions in regards to toxic nectar (Gegear et al. 2007, Liu et al. 2007). It should be noted that the concentrations of secondary compounds offered to pollinators in preference assays often exceed natural concentrations found in plant nectar (Adler and Irwin 2005, Wright et al. 2010, Adler and Irwin 2012, Mustard et al. 2012). Preference assays investigating the feeding response of pollinators to nectar relevant concentrations are required to better understand this dynamic.

Given the frequency with which toxic nectar occurs in angiosperms, the impacts of ingestion of these compounds on pollinator physiology are poorly studied. Nectar alkaloids may reduce nutrient assimilation in bird pollinators (Tadmor-Melamed et al. 2004), but no similar studies have been carried out using insect pollinators. To date, only four studies have investigated impacts of toxic nectar on pollinator fitness or reproduction. Negative impacts were observed in two experiments; the toxic alkaloid lupanine can decrease the reproductive success of Bombus terrestris microcolonies (Arnold et al. 2014), and the alkaloid gelsemine can reduce oocyte width in subordinate Bombus impatiens (Manson and Thomson 2009). However, the physiological impacts of nectar toxins may not always be negative. Two studies found that naturally occurring concentrations of nectar toxins have no impact on the survival or performance of bee offspring (Singaravelan et al. 2006, Elliott et al. 2008). Bee activity has also been shown to decrease after consumption of nectar toxins, but only at artificially high concentrations (Cook et al. 2013). Furthermore, nectar relevant concentrations of the alkaloid caffeine have been shown to increase the ability of honeybees (Apis mellifera) to learn traits associated with floral rewards (Wright et al. 2013). Finally, one recent study provides compelling support for the self-medication hypothesis (section 1.5.2); bumblebees infected with the gut trypanosome parasite Crithidia bombi that were fed a diet containing the nectar alkaloid gelsemine had parasite loads 2.2 times lower than control fed bees (Manson et al. 2010). If nectar toxins are commonly effective against pollinator parasites or pathogens,
pollinators may select for low concentrations of nectar secondary compounds, maintaining this trait in plant populations.

1.5.4 The role of plant secondary compounds in invasion biology

Invasive plants that express secondary metabolites in their floral nectar may still require visitation from mutualist pollinators in order to reproduce and spread (Stout 2007b, Stout 2007a). This has two noteworthy implications. First, if toxic nectar is a consequence of chemical defence of other plant tissues (pleiotropy hypothesis, see section 1.5.2), plant secondary compounds may play conflicting roles in the success of introduced, animal pollinated plants; they may increase success by suppressing plant competitors or herbivores (sensu novel weapons or enemy release), but also decrease success by reducing pollinator visitation (Strauss et al. 1999). It is therefore possible that the production of plant secondary compounds will be under simultaneous conflicting selection pressures. Second, similar to the novel weapons hypothesis, novel nectar toxins from an invasive plant could be more toxic to non-adapted native pollinators than pollinators from the plants’ invasive range. The potential for direct negative impacts of invasive nectar toxins for native pollinator health and the role of nectar secondary compounds in the invasion process clearly warrants further study.

1.6 Rhododendron ponticum as a study system

In the majority of my work, I used *Rhododendron ponticum* L. as a model species to study the impacts of plant invasion on native pollinators (Plate 3). *R. ponticum* provided a particularly useful experimental system, because although it is a successful invasive entomophilous plant in western Europe (Sheppard et al. 2005), it contains high concentrations of a plant secondary compound in its floral nectar (Koca and Koca 2007). I used this experimental system to investigate the impacts of plant invasion on native pollinators and also to question how nectar secondary compounds from an invasive plant impact native pollinators and plant-pollinator communities.
Plate 3. Invasive *Rhododendron ponticum*. Clockwise from top left, a pink-purple zygomorphic flower, an attractive inflorescence, a typical invaded Irish woodland (County Wicklow), nectar theiving ants feeding from holes left by nectar robbers in *R. ponticum*’s native range, *Bombus lucorum* simultaneously visiting a flower for nectar and pollinating. (Photo credit: Paul A. Egan).

*R. ponticum*, an evergreen, perennial shrub reaching up to 3 m in height and belonging to the family Ericaceae, shares its genus with approximately 1200 additional species (Colak et al. 1998). It spreads locally via vegetative reproduction, but requires sexually produced seeds for long distance dispersal (Cross 1975). It is a self-compatible plant, however higher levels of seed set occur following xenogamy (as opposed to autogamy or geitonogamy) (Stout 2007b) and autogamous self-pollination is partially prevented by temporal separation in the receptivity of stigmas and dehiscing of anthers (Mejias et al. 2002). *R. ponticum* was introduced to the British Isles as an ornamental species and as game cover in the late eighteenth century (Elton 1958) and subsequently became severely invasive (Plate 3, Cross 1982, Rotherham 2002). Invasive *R. ponticum* is ecologically and economically damaging, causing habitat loss and costs in terms of production losses in forestry and agriculture, and clearance costs (Dehnen-Schmutz et al. 2004).

Although now considered an introduced species in Britain and Ireland, *R. ponticum* was widely distributed in Europe during the Pleistocene period; interglacial pollen records demonstrate
that *R. ponticum* was present in Ireland, but retreated to more southern parts of Europe during glaciation periods (Watts 1966, Coxon and Waldren 1995). Currently its native distribution includes the Black Sea region (Turkey, Lebanon and Bulgaria, ssp. *ponticum*) and relict populations in the Iberian peninsula (Spain and Portugal, ssp. *baeticum*) (Colak et al. 1998, Ojeda et al. 2000, Mejias et al. 2002, Latorre and Cabezudo 2006), however molecular analysis of chloroplast and nuclear ribosomal DNA indicate that material in Britain and Ireland originated from Iberian populations (Milne and Abbott 2000). Although it is listed as one of the “Top 20” invasive weeds requiring biological control in its invasive range (Sheppard et al. 2005), *R. ponticum* is an extinction risk species in southern Spain and a Red Data Book species in Bulgaria (Colak et al. 1998, Ojeda et al. 2000). Nevertheless, in both ranges it dominates the habitat, forming dense stands and spreading aggressively throughout suitable environments (Rotherham 2001).

In Ireland, *R. ponticum* reduces native plant diversity through abiotic mechanisms (Cross 1982, Nadezhdina et al. 2004, Sutton and Wilkinson 2007). The attractive nature of its floral displays, however, indicates that it may also have biotic impacts on native plant-pollinator communities. *R. ponticum* produces large inflorescences containing 9-21 pink-purple zygomorphic flowers that produce large volumes of sugar-rich nectar (Plate 3, Stout et al. 2006). Mejias et al. (2002) observed 13 species of insects visiting *R. ponticum* flowers in Spanish populations, with large bees (*Bombus* and *Xylocopa*) making up more than half the visits. In native populations in Turkey, flowers are visited by wild honeybees (Colak et al. 1998, Silici et al. 2008), however in the invasive range, the majority of visits come from bumblebee species and hoverflies (Diptera; Syrphidae) and less from solitary bee and Lepidoptera species (Stout et al. 2006). Only medium to large-sized bees are large enough to reliably provide the service of pollination (Plate 3, Stout 2007a). Recent studies demonstrate density-dependent, indirect impacts of invasion on native plant pollination (Dietzsch et al. 2011) and insect-flower interaction networks (Stout and Casey 2014). Direct impacts on pollinators, however, remain largely untested.

For centuries, humans have been acutely aware that honey made from the nectar of some species of *Rhododendron* can cause “mad honey poisoning” (Gunduz et al. 2008). In fact, legend has it that poisoning from mad honey produced from *Rhododendron* led to the defeat of the Greek army of Xenophon in 400 B.C. (Silici et al. 2008). Mad honey poisoning occurs when humans ingest toxic diterpenes known as grayanotoxins (GTXs), polyhydroxylated, cyclic, nitrogen-free hydrocarbons that act on sodium channels in the central nervous system of
mammals (Koca and Koca 2007). In low doses, GTXs can cause dizziness, nausea or vomiting, and if enough is ingested, hypotension, bradycardia or even death (Onat et al. 1991). Recent studies have confirmed that GTX occurs at surprisingly high concentrations in the nectar and pollen of invasive *R. ponticum* (Koca and Koca 2007, Egan 2014). GTXs can negatively impact the physiology and behaviour of herbivores (El-Naggar et al. 1980, Klocke et al. 1991), but their effects on pollinators are poorly understood.

1.7 Research objectives

Although invasive species are considered one of the main drivers of pollinator decline, very little research considers how plant invasion impacts native pollinators; my thesis aims to address this knowledge gap. By utilizing an invasive study species that contains secondary compounds in its floral nectar, I also aimed to further our understanding of how nectar toxins impact pollinator health and behaviour. I pay special attention to the ways in which plant invasion impacts forage resources available to pollinators, and consider how plant traits (such as toxic nectar) may modulate the impacts of invasive plants for pollinators. My research takes place at a range of scales, from studies of pollinator individuals, to species, and finally entire plant-pollinator communities.

In Chapter 2, I first question whether individuals of a common bumblebee species are deterred by naturally occurring concentrations of *R. ponticum*'s nectar secondary compounds. Antifeedant properties resulting from toxic nectar could render the rewards provided by this invasive plant unsuitable for pollinators, and could ultimately have negative consequences for plant reproduction. I extend this study to investigate additional secondary compounds found in the floral nectar of other plant species. This allows for more general conclusions regarding the acuity of social bees species for the detection of plant secondary compounds.

Little is known about species-specific effects of plant invasion on native pollinators. The presence of secondary compounds in the nectar of *R. ponticum* may modulate the impacts of invasion for different pollinator species; those susceptible to the toxins will not be able to utilize the abundant resources provided by this plant, while tolerant species may rely on *R. ponticum* as an important forage resource. Chapter 3 investigates the effects of GTX consumption on the survival and behaviour of three ecologically and economically important native bee species. This chapter also investigates multiple drivers of pollinator decline simultaneously (e.g. invasion and parasite exposure/habitat loss) in order to better understand the impacts of interacting pressures on pollinators.
Even if *R. ponticum* provides a suitable replacement forage resource for some pollinators, it only flowers for part of the season. If native plant abundance and diversity is reduced by plant invasion, whole-season floral resource availability for pollinators may decrease. In Chapter 4, I take a community-level approach in order to investigate how seasonal variation in floral resources at sites invaded by *R. ponticum* impacts the structure of entire insect-flower interaction networks. This is also the first study to my knowledge to investigate an invasive plant that provides a floral resource to only a portion of the pollinator community in a network context.

Finally, invasion is a biogeographical phenomenon, thus comparative studies of invasive species in their native and invasive range could aid in our understanding of the impacts of invasion on native pollinator communities. Chapter 5 presents the first ever comparison of field replicated, quantitative insect-flower interaction networks in the native and introduced range of an invasive plant species. We use this biogeographical observational study to draw conclusions concerning the impacts of introduction of a novel plant species on plant-pollinator communities.

I finish with a synthesis of the overall findings, including a discussion of concurrent research on *R. ponticum* nectar toxins. I discuss methodological considerations, directions for future research and consider my findings in the broader context of the fields of invasion biology and pollinator decline.
Chapter 2

Bumblebees are not deterred by ecologically relevant concentrations of nectar toxins

2 Bumblebees are not deterred by ecological relevant concentrations of nectar toxins

2.1 Abstract

Bees visit flowers to collect nectar and pollen that contain nutrients and simultaneously facilitate plant sexual reproduction. Paradoxically, nectar produced to attract pollinators often contains deterrent or toxic plant compounds associated with herbivore defence. The functional significance of these nectar toxins is not fully understood, but they may have a negative impact on pollinator behaviour and health, and ultimately plant pollination.

This study investigates whether a generalist bumblebee, *Bombus terrestris*, can detect naturally occurring concentrations of nectar toxins. Using paired-choice experiments, we identified deterrence thresholds for five compounds found in the nectar of bee-pollinated plants: quinine, caffeine, nicotine, amygdalin and grayanotoxin. The deterrence threshold was determined when bumblebees significantly preferred a sucrose solution over a sucrose solution containing the compound. Bumblebees had the lowest deterrence threshold for the alkaloid quinine (0.01 mM); all other compounds had higher deterrence thresholds, above the natural concentration range in floral nectar. Our data, combined with previous work using honeybees, suggest that generalist bee species have poor acuity for the detection of nectar toxins. The fact that bees do not avoid nectar-relevant concentrations of these compounds likely indicates that it is difficult for them to learn to associate floral traits with the presence of toxins, thus maintaining this trait in plant populations.
2.2 Introduction

Pollination is a key ecosystem service provided by flower-visiting animals. It is estimated that over 87% of the world’s angiosperm species are animal pollinated and thus potentially influenced by pollinator foraging behaviour (Ollerton et al. 2011) because patterns of floral visitation by nectar- and pollen-collecting animals influence the quantity and quality of pollination events (Aizen and Harder 2007). In order to attract vital pollinators many plants produce sugar-rich nectar, the primary function of which is to reward animals for visiting flowers (Heil 2011). Nectar is the principle source of carbohydrates for most flower-visiting insects (Michener 1974, Nicolson 2011), however this reward can paradoxically contain low concentrations of potentially deterrent or toxic plant compounds. These secondary compounds, such as alkaloids, phenolics and non-protein amino acids, are produced in plant tissues as a means of chemical defence against herbivores (Baker and Baker 1975, Baker 1977, Adler 2000). Expression of toxins in nectar can be affected by herbivorous attack, and so the naturally occurring concentrations to which pollinators are exposed can fluctuate (Adler et al. 2006). Many adaptive functions have been proposed to explain the presence of these compounds in nectar (Table 1.1), including deterring nectar robbers (Janzen 1977, Baker et al. 1978), altering pollinator behaviour (Baker and Baker 1975, Rhoades and Bergdahl 1981, Ehlers and Olesen 1997, Wright et al. 2013) and providing antimicrobial properties that can benefit both the plant (by preserving the nectar quality for pollinators, Hagler and Buchmann 1993, Adler 2000), and the pollinators (by medicating against harmful pathogens and parasites, Manson et al. 2010). The functional significance of toxins in nectar is likely to depend on the ecological context and the nature of the toxin, but we still know relatively little about their influence on pollinators.

Understanding the significance that nectar toxins have on plant-pollinator interactions requires knowledge of how pollinators alter their behaviour in response to consumption of these compounds. For example, pollinators may avoid toxin-contaminated nectar: honeybees reject nectar containing nicotine, and several wild bee species avoid foraging on plants containing high concentrations of the alkaloid gelsemine (Detzel and Wink 1993, Hagler and Buchmann 1993, Adler and Irwin 2005). Occasionally, the opposite has been demonstrated: for example, free-flying honeybees prefer solutions containing low concentrations of the alkaloid caffeine, and were even found to increase visitation rates (Hagler and Buchmann 1993) or learn floral traits faster when it was present (Wright et al. 2013). However, most plant secondary compounds are toxic to animals (Rosenthal and Berenbaum 1992), and their
ingestion could represent a significant form of physiological stress that would require energy or resources to metabolise or cope with the toxin (Despres et al. 2007, Schuler 2011). If consuming such plant compounds is costly, one would predict that when nectar-feeders can detect toxins, they should learn to avoid plant species offering toxic nectar (Detzel and Wink 1993, Hagler and Buchmann 1993, Glendinning 2002, Adler and Irwin 2005). It remains unclear however, whether or not most pollinators can detect or are deterred by naturally occurring concentrations of secondary compounds in nectar. If these compounds do not deter pollinators, any benefit to the plant of their presence (e.g. the deterrence of nectar robbers (Janzen 1977) or suppression of nectar quality-altering microbes (Adler 2000) allow the trait to be maintained in the plant population.

Bumblebees such as the widespread species *Bombus terrestris*, are ecologically and economically important pollinators. They are generalists that visit many plant species, including those containing nectar toxins (Detzel and Wink 1993, Kretschmar and Baumann 1999, London-Shafir et al. 2003, Stout et al. 2006). Several studies have shown that when bumblebees and honeybees detect toxins such as the bitter-tasting alkaloid, quinine, they will learn to avoid floral traits associated with the compound’s presence in sucrose rewards (Chittka et al. 2003, Wright et al. 2010, Mustard et al. 2012). However, many of these studies use concentrations of toxins several orders of magnitude beyond their concentration in nectar. Whether or not bumblebees can detect the same compounds at concentrations encountered in floral nectar remains unknown.

Here, we performed a series of experiments to test whether *B. terrestris* was deterred by naturally occurring concentrations of nectar toxins in sucrose solutions. This study is the first to determine the deterrence thresholds of nectar toxins for a *Bombus* species. We discuss the resultant implications concerning bee gustatory acuity and bee health, as well as how our results add to the growing body of literature concerning the functional significance of toxins in nectar.
2.3 Materials and Methods

2.3.1 Subjects

*Bombus terrestris dalmatinus* (Linnaeus 1758) workers from four colonies (from Agralan Ltd, Swindon) were used for each secondary compound assay (total twelve colonies). Prior to use, colonies were maintained at 25-30°C in 24 h darkness and fed commercial pollen and Biogluc® (Agralan) bee food *ad libitum*.

2.3.2 Secondary compounds

Five compounds were investigated: quinine, caffeine, nicotine, amygdalin, and grayanotoxins (GTX) (see Table 2.1). With the exception of the compound quinine, and to large extent nicotine, these compounds are known to naturally occur in floral nectar of plant species foraged on by bees (Thomson and Goodell 2001, Roubik 2002, London-Shafir et al. 2003, Raguso et al. 2003, Tadmor-Melamed et al. 2004, Singaravelan et al. 2006, Stout et al. 2006). All of the compounds except for GTX I were supplied by Sigma-Aldrich (Dorset, UK). GTX (a mixture of GTX I and III) was isolated from flowers of *Rhododendron ponticum* L. from the UK using prep-HPLC. Flowers of *R. ponticum* were harvested from the Isle of Cumrae, Millport, Scotland and air dried. Dried flowers (100 g) were extracted into 1 L methanol at room temperature for 24 h. The extract was evaporated to dryness and redissolved in 500 ml water and partitioned with hexane (500 ml) twice. The water fraction was further partitioned with 300 ml chloroform four times and the chloroform partition was evaporated under reduced pressure to dryness, redissolved in 10 ml methanol and filtered through a 0.45 μm acrodisc. A 10 μl sample was diluted into 990 μl methanol and a 10 μl aliquot of this diluted sample was injected directly onto the liquid chromatography-mass spectrometry (LC-MS) system. LC-MS analysis was carried out using a Waters Alliance LC solvent delivery system with a ZQ MS detector on a Phenomenex Luna C18(2) column (150 X 4.0 mm i.d., 5 μm particle size) operating under gradient conditions, with A = MeOH, B = H2O, C = 1% HCO2H in MeCN; 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; column temperature 30°C and flow rate of 0.5 ml min⁻¹. GTX III was purchased commercially (Sigma-Aldrich, Dorset, UK) and used as a chromatographic standard to generate a calibration curve for this compound by quantification of the [M-H+formate]⁻ molecular ion in negative mode with m/z = 415.3 and eluting at 6.71 min. A second more abundant [M-H]⁻ ion with m/z=411.1 corresponded to the molecular weight of GTX I and eluted at 8.1 min. Using this method, the two GTXs were separated by over 1 min so they
could be purified from the fraction by HPLC by collecting fractions by time. HPLC was carried out using a semi-preparative Phenomenex Luna C18(2) column (150 x 10.0 mm i.d., 5μm particle size) operating under the same elution programme as described above but with an increased flow of 5ml min⁻¹ on a Waters Alliance LC system and a Waters fraction collector. Aliquots of 100 μL were injected directly onto the column, and the eluent was collected in 30 s batches and each collection was analysed directly by LC-MS as described above to determine the content. GTXs are diterpenoids with no chromophore, so they cannot be detected by their UV absorbance. Isolation of 4 ml of the methanol-soluble partition yielded 20 mg of the main compound (GTX I) and 1 mg GTX III identified earlier by comparison with an authentic standard. The major compound was evaporated to dryness and subjected to Nuclear Magnetic Resonance (NMR) spectroscopy. NMR spectra were acquired in MeOH-δ₄ at 30°C on a Bruker Avance 400 MHz instrument. Standard pulse sequences and parameters were used to obtain 1D ¹H and 1D ¹³C spectra. Chemical shift referencing was carried out with respect to internal tetramethylsilane at 0.00 ppm and verified as GTX I by comparison with published data (Burke and Doskotch 1990).

Nectar was collected from R. ponticum on the Isle of Cumbrae, Millport, Scotland. A 20 μL aliquot was diluted to 200 μL and injected directly on the LC-MS as described above, and the concentration of compounds present in samples from nectar were quantified in this nectar sample against calibration curves of authentic samples for both GTX I isolated here and commercial GTX III.

Quinine has not been reported in floral nectar, but it is widely used in behavioural studies of honeybees and bumblebees as an aversive stimulus (Chittka et al. 2003, Mustard et al. 2012), and is known to be repellent. We used it as a positive control. The concentrations at which the remaining secondary compounds occur in floral nectar have been previously determined (see Table 2.1), except for GTX, whose nectar concentration was determined in this study.
<table>
<thead>
<tr>
<th>Secondary compound</th>
<th>Compound class</th>
<th>Naturally occurring concentration in nectar (mM)</th>
<th>Plant species containing compound(^1)</th>
<th>Deterrence threshold exhibited by honeybees (mM)</th>
<th>Honeybee LD(_{50}) (^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>alkaloid</td>
<td>Unknown(^2)</td>
<td>unknown</td>
<td>10 mM (in 1.0 M sucrose)</td>
<td>LD(_{50})=0.62 mM (Toxicity of quinidine, a stereoisomer of quinine) (Detzel and Wink 1993)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>alkaloid</td>
<td>0.003 mM-0.253 mM (Wright et al. 2013)</td>
<td>Coffea canephora, Coffea arabica, Coffea liberica, Citris paradisi Citrus maxima, Citrus sinensis, Citrus reticulata</td>
<td>10 mM (in 1.0 M sucrose) (Mustard et al. 2012)</td>
<td>LD(_{50})=102 mM (Detzel and Wink 1993)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>alkaloid</td>
<td>0-0.015 mM (0-2.5 ppm) (Detzel and Wink 1993, Tadmor-Melamed et al. 2004)</td>
<td>Nicotiana tabacum, Nicotinia glauca</td>
<td>NA</td>
<td>LD(_{50})=12.3 mM (Detzel and Wink 1993)</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>cyanogenic glycoside</td>
<td>0.009-0.015 mM (4-7 ppm) (London-Shafir et al. 2003)</td>
<td>Amygdalus communis</td>
<td>10 mM (in 1.0 M sucrose) (Wright et al. 2010)</td>
<td>LD(_{50})=0.066 mM (Detzel and Wink 1993)</td>
</tr>
<tr>
<td>Grayanotoxin</td>
<td>diterpene</td>
<td>0.07 mM</td>
<td>Rhododendron ponticum</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^1\) This is not a comprehensive list of plant species containing these compounds; it includes only plants species used to determine the concentration of compounds in nectar/pollen in the references listed. \(^2\) The nectar of plants containing quinine in other tissues (bark, leaves, roots) has not been analyzed for the presence of secondary compounds. \(^3\) LD\(_{50}\) results from oral acute toxicity tests. NA = not applicable.
2.3.3. Experimental protocol

We determined the deterrence threshold for each secondary compound using a paired choice assay in which bumblebees were offered two sucrose solutions, one with and one without the compound at a variety of concentrations. Sucrose solutions (0.5 M, within the range found in the nectar of bee-pollinated flowers (Baker 1975)) were made by mixing grade II sucrose (Sigma Aldrich, Dorset, UK) with deionised water. Serial dilutions were performed to obtain different concentrations of each secondary compound (range of 0.001 mM-10 mM, encompassing the naturally occurring concentrations of the compounds in floral nectar (Detzel and Wink 1993, Kretschmar and Baumann 1999, London-Shafir et al. 2003, Tadmor-Melamed et al. 2004, Wright et al. 2013)), depending on the toxicity and availability of each compound.

Worker bumblebees from each colony were removed and placed into individual plastic containers. Nest bumblebees (spending most of their time caring for brood inside the nest, never foraging) were avoided by refraining from using the smallest workers (Goulson et al. 2002). Bees were chilled on ice for approximately 3 min or until movement slowed, measured (body length, thorax and abdomen width) and weighed, and randomly allocated to a toxin concentration. Each bee remained in a separate container and was allowed to acclimate for at least 1 h. Forty bumblebees, ten from each of four colonies, were allocated to each of the concentrations of each compound.

Assays were conducted in 650 ml plastic containers (160x110x45 mm) with lids containing 1 mm diameter ventilation holes. The containers had three additional 10 mm diameter holes on three of the four sides where feeding tubes could be inserted horizontally. Feeding tubes were 3 ml centrifuge tubes with four 2 mm holes: bees could alight on the tubes and feed from the openings. Bees were given a choice between two solutions: a 0.5 M sucrose solution (internal control), and an identical 0.5 M sucrose solution containing the toxin. Bees were also supplied with a third tube containing deionised water. Tubes were weighed prior to being inserted into the container and the bee was left to feed for 24 h in growth cabinets at 28°C, 60% relative humidity and 24 h darkness, mimicking nest conditions (Heinrich 2004). Feeding tubes were then reweighed and the amount of food consumed from each was calculated. Identical setups containing no bees were used daily to control for the change in tube weight due to evaporation (external controls) and the consumption per bee (g) was adjusted accordingly. At least eight of these control setups were run for each concentration of each compound. Data from individual bumblebees were only used in the analysis if bees were still alive at the end of the 24 h test period.
Forty control bumblebees were fed 0.5 M sucrose in both tubes (ten from each of four colonies) for comparison with bees fed toxins.

2.3.4. Data Analysis

Consumption data for each of the six compounds were analysed using generalised linear models (GLMs) with repeated measures. Concentration and solution type (presence/absence of the toxin) were included in the model as main effects and a significant interaction between the two indicated the presence of a deterrence threshold for a given compound. A least significant difference (LSD) post hoc comparison was used for all pairwise comparisons. Total consumption (cumulative consumption by each bumblebee, both the internal control and the solution containing the toxin) was compared between secondary compounds using concentrations for which the design was fully factorial (the three lowest concentrations tested, 0.001 mM, 0.01 mM and 0.1 mM) using GLMs. Logistic regression was utilized to determine whether there was a significant effect of toxin on mortality. All analyses were carried out using the statistical package SPSS Statistics®, version 20 (IBM).

2.4 Results

2.4.1. Bumblebees are not deterred by naturally occurring concentrations of nectar toxins

Bumblebees failed to be deterred by any of the compounds tested (nicotine, amygdalin, caffeine and GTXs) at naturally occurring concentrations in nectar (Figure 2.1). In contrast, the alkaloid quinine was readily avoided even at doses as low as 0.01 mM (Figure 2.1a, GLM, $\chi^2 = 59.2, P < 0.001$). The pairwise comparison illustrated that bumblebees preferred the pure sucrose solution (the internal control) over a quinine concentration of 0.01 mM ($P < 0.001$), and continued to exhibit this preference for the two highest quinine concentrations (Figure 2.1a).

By contrast, bumblebees had higher deterrence thresholds for the other alkaloids. While nicotine was deterrent at 0.1 mM (Figure 2.1b, GLM, $\chi^2 = 20.2, P < 0.001$), in tobacco flower nectar it has been found at concentrations of 0.015 mM (Tadmor-Melamed et al. 2004), nearly seven times lower than the deterrence threshold of *B. terrestris*. The preference of the bumblebees for the pure sucrose solution continued for the 1 mM nicotine concentration, but surprisingly individuals fed the highest concentration of nicotine, 10 mM, did not show a preference for either solution ($P = 0.974$). They did however consume less total food than
individuals fed any of the four lower concentrations (Figure 2.1b, $F = 3.44 \, P = 0.010$). The deterrence threshold for another nectar alkaloid, caffeine, was 10 mM and was the highest of all the compounds we tested (Figure 2.1d, GLM, $\chi^2_3 = 10.0 \, P < 0.01$). This value is 20 times higher than the highest caffeine concentration found in floral nectar, 0.5 mM (Kretschmar and Baumann 1999), and three orders of magnitude higher than the deterrence threshold for the alkaloid quinine.

The bumblebees' deterrence threshold for the cyanogenic glycoside, amygdalin, was 1 mM (Figure 2.1c, GLM, $\chi^2_3 = 3.8 \, P < 0.05$) - more than 60 times greater than the highest concentration of amygdalin found in floral nectar (0.015 mM) (London-Shafir et al. 2003). Finally, bumblebees were not deterred by GTX in any of the concentrations we tested (Figure 2.1e, GLM, $\chi^2_3 = 0.604 \, P = 0.739$).
Figure 2.1. Mean (± s.e.m.) consumption by Bombus terrestris of 0.5 M sucrose solution, with (light grey bars) or without (dark grey bars) one of five nectar toxins. Where bars are missing, assays were not completed because of limited availability of compounds. Asterisks indicate significant differences between consumption of two solutions at a given concentration according to (LSD) post-hoc comparisons (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). Black arrows represent naturally occurring concentrations of the compound in floral nectar.
2.4.2. Compensative feeding does not occur for all nectar toxins

The total amount of food consumed (sucrose solution + sucrose solution containing toxic compounds) by bumblebees differed significantly depending upon which toxin was consumed (Figure 2.2a, GLM, $\chi^2 = 70.3$, $P < 0.001$). The total consumption of individuals fed solutions containing caffeine, nicotine and GTX was significantly lower than that of the control bumblebees ($P < 0.001$, $P = 0.002$, and $P < 0.001$ respectively). By contrast, the total consumption of bumblebees fed quinine and amygdalin did not differ from control bumblebees ($P = 0.244$ and $P = 0.803$ respectively). The analysis of total food consumption was undertaken for the lowest concentration of toxin tested, 0.001 mM, because bumblebees could not detect any of the toxins at this level. However, the same pattern was found when all concentrations for which the design was fully factorial across all toxins were analyzed, (0.001 mM, 0.01 mM and 0.1 mM): bumblebees fed caffeine, nicotine and GTX consumed significantly less total food than controls (GLM, $\chi^2 = 30.3$, $P < 0.001$).

The toxins also had a significant effect on bumblebee mortality within a 24 hour time period (GLM, $\chi^2 = 15.9$, $P = 0.007$, all concentrations included). Bumblebees fed amygdalin and caffeine had significantly higher mortality rates than individuals fed any of the other compounds or control bumblebees (Figure 2.2b, $P = 0.027$ and $P = 0.045$ respectively). Survival of the bees fed GTX, nicotine, or quinine did not differ from that of the control bumblebees.

Figure 2.2. Mean (± s.e.m.) a. total consumption of solutions at lowest concentration (0.001 mM) for each nectar toxin, and b. mortality of *Bombus terrestris* fed five different nectar toxins. Control bumblebees were fed 0.5 M sucrose in both solutions so had no exposure to any toxin. N= 40 bees/toxin/concentration. Lower case letters represent significant ($P < 0.05$) differences in total consumption between compounds according to least significant difference (LSD) post hoc comparison.
2.5 Discussion

Our experiments show that bumblebees are not deterred by a variety of naturally occurring levels of nectar toxins. This finding has important implications for bumblebee health and for plant-pollinator interactions among Bombus-pollinated plants that produce toxins in their nectar, such as Rhododendron (containing GTX) (Stout et al. 2006) and almond tree species (containing amygdalin) (Thomson and Goodell 2001). Because the compounds we tested did not have repellent effects on bumblebees at nectar relevant concentrations, these pollinators are unlikely to alter their behaviour to avoid flowers with such compounds.

2.5.1 Bees have poor acuity for toxins in nectar

Our data, combined with those from previous studies using honeybees, demonstrate that generalist bees have relatively low sensitivity for plant toxins in sucrose solutions. Previous work has determined honeybee deterrence thresholds for caffeine, quinine and amygdalin. This work has consistently found that honeybees do not respond to levels of these compounds less than 10 mM (Wright et al. 2010, Mustard et al. 2012). For caffeine, the deterrence threshold concentrations for honeybees and bumblebees are similar; however, bumblebees were more sensitive to amygdalin and quinine in our assays (deterrence thresholds of 1 mM and 0.01 mM, respectively). Other insect taxa have greater gustatory acuity for these compounds; fruit flies, for example, have deterrence thresholds for caffeine and quinine that are 10-100 times lower than those of bees (Sellier et al. 2011). Similarly, gypsy moth larvae (Lymantria dispar (L)) are deterred by caffeine at levels 100 times lower than bees (Sheilds et al. 2008).

Generalist bee species may have poor acuity for the detection of toxins in nectar because they have few gustatory receptors (Grs) that can detect these compounds. For example, the honeybee genome encodes only 10 orthologous genes for g-protein coupled Grs (Robertson and Wanner 2006). This is in contrast to Dipteran species such as fruit flies and the mosquito, Anopheles gambiae, which have many more genes for Grs (flies: 68, A. gambiae, 76) (Dunipace et al. 2001, Scott et al. 2001, Hill et al. 2002, Robertson et al. 2003). The greater relative diversity of Grs in flies and other insects probably reflects stronger selection for the detection of toxins in food in these species (Robertson and Wanner 2006).

It is possible that natural selection for the ability to detect plant toxins has not been strong enough to force diversification of eusocial bee’s Grs to improve gustatory acuity for these chemicals. This may be a consequence of eusociality, where individual bees are the
consumers, but selection pressures act on the colony as the reproductive unit. In solitary animals, the individual bears the fitness cost of toxin consumption. In eusocial honeybees and bumble bees, foragers collect food for the entire colony. If a forager ate nectar contaminated with toxins that it could not detect, it might die, but with little impact on the fitness of the colony [though more impact on bumblebees as compared with honeybees, because of their relatively small colonies (Khoury et al. 2011).] Selection for the ability to detect toxins would only occur when the queen, and therefore the fitness of the colony, was affected by toxins in nectar.

Our results indicate that out of the classes of toxic compounds tested, individuals of the species *B. terrestris* are relatively good at detecting and avoiding alkaloids. However, even within this specific class of compounds the deterrence thresholds varied across four orders of magnitude for different chemicals (e.g. caffeine, nicotine and quinine). Alkaloids are one of the most common and chemically diverse groups of plant compounds, with more than 12,000 structures described (Wink 1993). The common frequency with which alkaloids are found in higher plants and their toxicity has led insects to develop the ability to detect and reject these chemicals in their food. The diverse chemical structures within alkaloids, however, makes some easier to detect than others.

### 2.5.2 Total consumption of solutions is affected by toxins in nectar

Our results indicate that when bumblebees consume low, nectar relevant doses of caffeine, nicotine and GTXs, their total intake of food was depressed, regardless of whether they could readily distinguish the two solutions. A study on *D. melanogaster* found the same phenomenon: flies ate less total sucrose solution when the alkaloids lobeline, nicotine and strychnine were present (Sellier et al. 2011). This reduction in intake of all solutions after toxin consumption may be due to post-ingestive detection of the toxins, which is modulating appetite (Wright et al. 2010). In addition, in our study, bumblebees fed the 10 mM nicotine solution consumed equal, but very small, amounts of both solutions, even though their deterrence threshold was at a lower concentration (0.1 mM, Figure 2.1b). Consumption of this concentration of nicotine could have damaged chemosensory sensilla or gustatory receptor neurons of individuals, preventing them from detecting nicotine even though they were capable of doing so at lower concentrations (0.1 mM) (Sellier et al. 2011).

Bumblebee colonies must reach a minimum size in order to produce new queens and males (Muller and Schmid-Hempel 1992). If consumption of toxins in floral nectar causes appetite
suppression in foraging workers, colonies may not reach this crucial point as early in the season or at all. This could result in a decrease in queen and male production, and because bumblebees have an annual life cycle could have a substantial population-level effect (Gill et al. 2012, Henry et al. 2012, Whitehorn et al. 2012).

2.5.3 Functional significance of nectar toxins

Bumblebees are generalist pollinators, and based on the large percentage of plants that have toxins in their nectar (Baker and Baker 1975, Baker et al. 1978) it is likely that bumblebees encounter these kinds of toxins often (Stephenson 1982, Adler and Irwin 2005, Stout et al. 2006). It is possible that legitimate pollinators such as bumblebees have therefore selected for concentrations of toxins in floral nectar that remain below their deterrence level (Wright et al. 2013). For example, if a honeybee learns to associate floral traits with bad-tasting nectar, it will avoid flowers with these traits (Wright et al. 2010) and will potentially communicate the poor quality of the nectar to other colony members or not recruit them to this food source (Tan et al. 2012). In this way, individual bees could drive natural selection towards concentrations of these compounds in nectar that are below their deterrence threshold (Wright et al. 2010, Wright et al. 2013).

Our data suggest that in the field, low levels of toxic compounds in nectar do not affect bumblebee foraging behaviour. These findings are in contrast to those of a similar study investigating the gustatory responses of bumblebees in response to different sugars, where nectar relevant concentrations and sugar identity were shown to impact bumblebee preference (Mommaerts et al. 2013). Bumblebee-pollinated plants containing toxic compounds in their nectar would not suffer from reduced pollination, thus allowing this plant trait to be maintained if it conferred any fitness benefit to the plant. Selection for the production of toxins in nectar is likely to be the result of other factors affecting nectar secretion and production, such as nectar robbery, damage from herbivores, or reduction of nectar quality due to microorganisms. For example, nectar toxins could be toxic or deterrent to nectar thieves but not deter legitimate pollinators; thus they act in a similarly selective manner to morphological characters such as sticky peduncles or narrow corolla tubes (Janzen 1977, Stephenson 1982).
2.5.4 Conclusions

This is the first assay to report that the deterrence thresholds of bumblebees are well above nectar relevant concentrations of toxic compounds in Bombus-pollinated plants. Our data are also the first to provide concentrations that inhibit feeding of the bumblebee for some chemicals commonly found in floral nectar, and to indicate that the acuity of this generalist bumblebee for nectar toxins is poor in comparison to other insect species. This work adds to the growing body of research on the functional significance of nectar toxins on plant-pollinator interactions and the impacts of these chemicals on bee health.
Chapter 3

Toxic nectar modulates impacts of invasive *Rhododendron* for native bees

3 Toxic nectar modulates impacts of invasive *Rhododendron* for native bees

3.1 Abstract

The impacts of invasive alien plants on native pollinators are poorly understood. On the one hand, plant invasion may reduce the diversity and/or abundance of native flowering species that pollinators rely on for food. Conversely, invasive flowering plants may present an abundant supplemental forage resource for pollinators, providing their rewards are suitably nutritious and palatable. Many plants, however, contain defensive secondary compounds not only in their vegetative tissues, but also in floral nectar. Nectar secondary compounds can impact pollinator health and behaviour, and if present in invasive plants, may modulate the impacts of plant invasion for native pollinators. We tested three ecologically and economically important native bee species for acute and chronic responses to consumption of grayanotoxins (GTXs), which occur in the nectar of invasive *Rhododendron ponticum*. Using non-choice bioassays, we examined the effect of GTX consumption on bee survival and behaviour in isolation and also in combination with additional natural stressors encountered frequently by wild pollinators (parasite infection and food stress).

GTX consumption resulted in rapid and acute mortality for the honeybee, *Apis mellifera*. The solitary mining bee, *Andrena carantonica*, exhibited no lethal effects but was deterred from feeding on GTX solutions, and showed sub-lethal effects including malaise behaviours after consumption. In contrast, GTX consumption had no negative impacts on survival or behaviour of the bumblebee *Bombus terrestris*, even when individuals were simultaneously exposed to parasite infection and food stress. Our study demonstrates that invasive *R. ponticum* provides a supplemental forage resource only to pollinators that can tolerate GTXs. Consequently, specific traits of invasive plants such as toxic nectar can modulate the impacts of plant invasion for pollinators. Ultimately, nectar toxins may act as a hidden barrier to invasion for entomophilous plants that must be overcome by establishing mutualisms with tolerant pollinators.
3.2 Introduction

Invasive alien plants are drivers of global environmental change and can severely impact ecosystems by affecting native biodiversity and ecosystem processes (Stout 2011, Simberloff et al. 2013). One ecosystem process that has the potential to be affected by invasive plants is pollination. Invasive species are considered a key driver of pollinator decline (Potts et al. 2010b, Gonzalez-Varo et al. 2013), yet little research has investigated the direct impacts of invasive plants on native pollinators (Stout and Morales 2009).

Invasive alien plants may indirectly or directly impact native pollinator behaviour, populations and/or communities (Bjerknes et al. 2007, Aizen et al. 2008b, Moroń et al. 2009). The direction of impacts is likely to depend on how invasion influences resources essential to pollinators such as forage resources. Plant invasion can reduce forage resource availability for pollinators by decreasing the diversity or abundance of native flowering species (Martin 1999, Cox and Elmqvist 2000). Such changes to native plant community composition could affect pollinator community structure (Aizen et al. 2008b). Conversely, entomophilous, mass-flowering invasive plants may provide native pollinators with an abundant alternative forage resource, especially in areas with few native flowers (Graves and Shapiro 2003). These novel rewards may mitigate the loss of native co-flowering plants, and could even increase pollinator carrying capacity, assuming that they are spatially and temporally available (Chittka and Schürkens 2001, Tepedino et al. 2008, Nienhuis and Stout 2009).

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 and Morales 2009, Kaiser-Bunbury et al. 2011). Some floral nectar is known to be toxic or unpalatable to pollinators (Pryce-Jones 1942, Majak et al. 1980) due to the presence of secondary compounds, such as alkaloids, terpenes, or phenolics, usually associated with defence against herbivores (Adler 2000). Nectar secondary compounds are geographically and phylogenetically widespread, they are found in species in at least twenty-one different plant families (Adler 2000). However their impacts on pollinators are poorly understood (Cook et al. 2013, Manson et al. 2013). Toxic or deterrent nectar in an invasive plant could be particularly harmful for non-adapted native flower-visited (C.f. novel weapons, Callaway and Ridenour 2004), and thus has the potential to modulate the impacts of plant invasion for pollinators.

Secondary compounds in floral nectar tend to occur at low concentrations in comparison to other plant tissues (Adler and Irwin 2012, Manson et al. 2012), and thus rarely have acute
lethal effects for pollinators (but see Pryce-Jones 1942, Majak et al. 1980). However, even if survival is unaffected by exposure to a chemical, insects may experience sublethal effects resulting in decreased growth or fecundity (Desneux et al. 2007, Cresswell 2011). Indeed, consumption of nectar secondary compounds can affect pollinator physiology (Tadmor-Melamed et al. 2004, Manson and Thomson 2009) and behaviour (Wright et al. 2010, Cook et al. 2013, Manson et al. 2013, Wright et al. 2013), but impacts are dose dependent and may only be apparent at concentrations higher than those naturally encountered in floral nectar. In addition, pollinators are often simultaneously exposed to multiple stressors, including parasite infection and food stress (Goulson et al. 2008, Gonzalez-Varo et al. 2013). Even if nectar secondary consumption does not result in acute or sublethal effects in isolation, when combined with additional stressors, negative impacts on pollinator health may be realized (Brown et al. 2000, Manson et al. 2010, Vidau et al. 2011). While the impacts of multiple stressors on pollinators are recognized as causing potentially additive or synergistic effects (Vanbergen and Insect Pollinators Initiative 2013), few studies have tackled this issue.

*Rhododendron ponticum* subsp. *baeticum* was introduced from the Iberian peninsula into Britain and Ireland in the eighteenth century and subsequently became one of the most invasive plant species in both islands (Cross 1975). Mature plants produce hundreds of flowers clustered on dense inflorescences containing copious volumes of sugar-rich nectar, making plants attractive to native flower-feeding insects, particularly bees (Cross 1975, Stout et al. 2006). Despite its reliance on these insects for pollination (Stout 2007b), the nectar of *R. ponticum* contains high concentrations of potentially toxic diterpenes known as grayanotoxins (GTXs) (Tiedeken et al. 2014). 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.

This aim of this study was to investigate how nectar secondary compounds from invasive *R. ponticum* impact native pollinators, in order to aid understanding of the overall impacts of invasion by this plant species on native pollinator health and well-being. We focused on impacts on native bees because *R. ponticum* is primarily bee-pollinated (Mejías et al. 2002, Stout et al. 2006), bees are important pollinators both ecologically and economically (Morse and Calderone 2000, Gallai et al. 2009, Garibaldi et al. 2013) and because bees are in decline worldwide (Biesmeijer et al. 2006, Cameron et al. 2011). Using a series of laboratory-based, non-choice bioassays we specifically tested the following hypotheses:
Hypothesis 1: GTXs in the nectar of invasive *Rhododendron* have lethal effects on native bees.

Hypothesis 2: In the absence of lethal effects, GTX ingestion causes sub-lethal changes in food consumption and behaviour.

Hypothesis 3: GTX consumption exacerbates effects of parasite infection/food deprivation.

### 3.3 Materials and Methods

#### 3.3.1 *Rhododendron ponticum* nectar collection and artificial nectar preparation

In total 48 ml of floral nectar was collected from approximately 5,400 *R. ponticum* flowers from four populations in Ireland (Appendix C, Table C.1), pooled into one sample, and stored at -80 °C. Twenty samples were diluted 200 fold with nanopure water for analysis of sucrose, fructose and glucose content (Appendix C, Figure C.1) using high-performance liquid chromatography (HPLC) on a Dionex DX500 HPLC system using an ED40 electrochemical detection unit. The mobile phase was 100 mM NaOH. Twenty µl of this sample was injected on to a Carbopack PA-100 column (Dionex, Sunnyvale, California, USA). Sugars were eluted isocratically with 100 mM NaOH with a flow rate of 1 ml/min. Elution profiles were analysed with Chromeleon software (Thermo Fisher Scientific).

GTX in *R. ponticum* nectar is comprised of GTX I and III, however GTX I is the dominant component (Egan 2014). Total GTX content in our composite *R. ponticum* nectar was 0.44 µg GTX per milligram fresh weight nectar (determined using the methodology previously described in Tiedeken et al. 2014). This value is consistent with mean GTX concentrations from *R. ponticum* populations in Ireland (Egan 2014), and was used in our artificial nectar (Table 3.1). GTX for use in artificial nectar was isolated from *R. ponticum* floral material collected from the same populations as nectar (Table C.1, see Appendix C.1 for isolation methodology). Up to five treatment solutions were used in assays depending upon the biology and availability of bee species (Table 3.1). To create artificial nectar treatment solutions, we mixed sugars with deionised water to obtain an artificial nectar base simulating the sugar concentration of *R. ponticum* nectar: 0.61 M sucrose, 0.13 M fructose and 0.06 M glucose (Figure C.1). GTX was dissolved to the nectar-relevant concentration by adding warm (<50°C) artificial nectar. For solutions used to calculate honeybee LC_{50}, serial dilutions were carried out using the artificial nectar base. All solutions were prepared and immediately stored at -80 °C until ready for use. Samples of final solutions were analyzed to verify GTX concentrations.
Table 3.1. Five treatment solutions used in bee assays. Due to differences in bee biology or availability, not all treatments were utilized in all assays.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment description</th>
<th>GTX concentration</th>
<th>Assays in which treatment was utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td><em>R. ponticum</em> nectar</td>
<td>0.44 µg/mg</td>
<td>Honeybee, bumblebee restrained and unrestrained</td>
</tr>
<tr>
<td>T2</td>
<td>Artificial nectar control-contains no GTX, but mimics <em>R. ponticum</em> sugar content</td>
<td>0 µg/mg</td>
<td>Honeybee, bumblebee restrained and unrestrained and solitary bee unrestrained</td>
</tr>
<tr>
<td>T3</td>
<td>Artificial nectar + GTX I and GTX III</td>
<td>0.44 µg/mg</td>
<td>Honeybee, bumblebee restrained and unrestrained and solitary bee unrestrained</td>
</tr>
<tr>
<td>T4</td>
<td>Artificial nectar + GTX I</td>
<td>0.44 µg/mg</td>
<td>Honeybee, bumblebee restrained and unrestrained</td>
</tr>
<tr>
<td>T5</td>
<td>Artificial nectar + GTX III</td>
<td>0.096 µg/mg</td>
<td>Honeybee, bumblebee restrained and unrestrained</td>
</tr>
</tbody>
</table>

1Concentration is expressed in µg GTX per milligram fresh weight nectar. GTX I and III are both found in the nectar of *R. ponticum*, so treatment 3, which contained them in their natural ratios, 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. 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.

3.3.2 Bee species

We used three bee species in this study that are native to habitats invaded by *R. ponticum*; the honeybee, *Apis mellifera mellifera*, a bumblebee species, *Bombus terrestris audax* and a solitary mining bee, *Andrena carantonica*.

**Honeybees**: In 2012, honeybees were obtained from two free foraging, disease-free colonies at the Trinity College Dublin Botanic Gardens, Dartry, Dublin. During the study, queens were not changed, colonies were not treated for *Varroa*, and appeared in good health.

**Bumblebees**: Bumblebee colonies were obtained from a commercial supplier (Koppert, The Netherlands). Upon arrival, we ensured colonies contained live queens, were still producing worker brood and screened for parasites by examining faecal samples from 10 workers per colony under a phase contrast microscope (x 40 magnification). All colonies were maintained at 25-30°C and 24 h darkness to mimic nest conditions (Heinrich 2004) and fed *ad libitum* commercial pollen and Biogluc® (Koppert) bee food until bees were ready for use in assays.

**Solitary bees**: Female solitary bees were collected from an aggregation on a south-facing incline at Trinity College Dublin in spring, 2013. Bees returning to their nests after foraging
were collected in plastic vials on warm sunny days (>15 °C, wind speed <4 on Beaufort wind scale) and brought back to the lab to acclimate before being used in the study.

3.3.3 Hypothesis 1: Effects on native bee survival

Honeybees

Pilot experiments demonstrated that free-flying honeybees would not feed on GTX solutions, so restrained honeybees were used. The morning before assays began approximately 250 foraging honeybees were collected in plastic vials (2.5 cm diameter) as they returned to their colonies. Individuals were chilled on ice until movement ceased and were restrained using plastic harnesses (made out of a 1 ml pipette tip with the tip cut off, 4 cm in height) and secured using a thin strip of duct tape behind the head (Bitterman et al. 1983). Care was taken to ensure that the bee's head and neck were unobstructed. Harnessed bees were immediately fed 5 μL 50% Apinvert solution (Bee supplies, Sandford, Dublin), allowed to acclimate for 1 h and then fed 4 additional 5 μL Apinvert drops. If bees showed an unreliable proboscis extension response (PER), they were excluded from experiments. Bees were left overnight in climate controlled chambers (Adaptis, Conviron™) at 25°C, 70% relative humidity, 0 light (Seeley and Heinrich 1981). The next morning, 50 bees were randomly assigned to one of five treatments (see Table 3.1) 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.

A similar assay was performed to calculate a dose-response relationship. Oral toxicity tests were based on the US Environmental Protection Agency (USEPA) guidelines for the acute toxicity testing of honeybees (USEPA 1996). Bees from one colony were caught and harnessed using the above methods, and fed 4 x 5 μL drops of one of six treatments: 0.44 μg/mg GTX, 0.22 μg/mg GTX, 0.11 μg/mg GTX, 0.055 μg/mg GTX, 0.0275 μg/mg GTX and treatment 2 (Table 3.1) which contained no GTX (n=30 bees per level, divided equally into 5 trials). Six hours, 24 h and 32 h after dosing, bees were fed 50% Apinvert solution ad lib. Survival was recorded at 24 and 48 h for the calculation of LC_{50} values.

Bumblebees

In order to directly compare bumblebee and honeybee responses to GTX, an assay with restrained bumblebees was performed (see Appendix C.2). However, because we wanted to test for chronic exposure to GTX, long-term assays were also carried out with unrestrained
bumblebees. Workers from each of three *B. terrestris* colonies were removed and placed into plastic vials, taking care to avoid nest bees. Workers were weighed and measured, and randomly allocated to one of the five treatments (Table 3.1). Bees were transferred to 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 could be inserted horizontally. Bees could alight on the feeding tube, comprised of a 0.75 ml centrifuge tubes with four 1.5 mm holes, and feed. A dish (0.5 cm diameter) containing commercial pollen (3.2 g ± 0.34 g) (Koppert) was provided at the start of the assay. All five treatments (Table 3.1) were fed to the bees for seven days (n= 6 bees for treatment 1, n=12 bees for treatments 2-5). Because individual bumblebees consume large volumes and 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* in its invasive range (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.

**Solitary bees**

To our knowledge, *A. carantonica* has not been harnessed in experiments and because we had a limited number of individuals, we only carried out the 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). All bees were fed 50% Apinvert solution ad libitum during the first 24 h to ensure they were equally satiated and allow acclimatization. Bees were kept in a growth cabinet (20 °C, 60% relative humidity, 12:12 dark/light setting), and on day two, treatment solutions were supplied in feeding tubes and survival was recorded daily for 30 days.

**3.3.4 Hypothesis 2: Sublethal effects**

**Honeybees**

Because honeybees were a) harnessed and b) demonstrated an acute lethal response to nectar GTXs (Figure 3.1), behavioural responses and consumption of treatment solutions were not measured.

**Bumble and solitary bees**
In order to record differences in the behaviour 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: (1) Exploring, (2) Still/resting, (3) Consumption of treatment solution, (4) Pollen manipulation, (5) Grooming, (6) Flying, (7) Distress behaviours: bees exhibited two distinct behaviours that appeared to indicate distress or toxicity; a) Paralysis; individuals laid on backs, legs twitching, or b) Intensive grooming: individuals rubbed their hind legs together continuously for 10 s or more (as described in Hurst et al. in press).

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 (in grams). We kept daily evaporation controls, and tracked differences between consumption for bees fed the control solution (treatment 2) and the solution containing GTX (solution 3) over the entire 30-day period.

3.3.5 Hypothesis 3: Additional stressors

Bumblebees did not exhibit any lethal or sub-lethal effects and (as the main legitimate pollinators of *R. ponticum* in its invasive range) were used in the assessment of additional stressors.

*Parasite assay*

In March 2013, we collected 74 *B. terrestris* queens emerging from hibernation in two locations in Dublin City, Merrion Square and the National Botanic Gardens. Queens were screened for *Crithidia bombi* infection by collecting and viewing a faecal sample under a phase-contrast microscope (40x magnification). Infected queens were kept at 25-30°C and 24 h darkness and fed *ad libitum* commercial pollen and Apinvert bee food. To make an inoculum, faecal samples from 12 infected native queens were mixed and diluted with 50% Apinvert to a concentration of 2500 *Crithidia* cells/μL (determined using a Neubaumer Improved Haemocytometer, VWR, Ireland) (Schmid-Hempel and Schmid-Hempel 1993, Logan et al. 2005). Thirty stock workers from each of three *B. terrestris* colonies were removed from the colony, starved for 2 h and fed with 10 μL of standard inoculum. Workers were kept in wooden boxes (10 cm x 7 cm) for 10 days and fed *ad libitum* pollen and 50% Apinvert until they reached peak infection, which previous experiments have revealed occurs at this time point (Otterstatter and Thomson 2006). A *Crithidia* inoculum was then created for each colony by harvesting faecal samples from that colony's stock workers and diluting as above. Sixty
additional workers were removed from each colony and infected with *Crithidia* using the colony-specific inoculum. These workers were randomly divided into two groups, kept individually in the same set up used for 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.

Survival under stress assay

Forty bees from each of three *B. terrestris* colonies were transferred to individual boxes (same as used for survival assays) and randomly assigned to either the control treatment (treatment 2) or the treatment containing GTX I and III (treatment 3), n= 60 bees per treatment. The 0.75 ml feeders used in the survival experiment were filled with treatment solutions and the weight was recorded before exposing the feeder to the bee. Ten empty boxes were set up as evaporation controls. Bees were allowed to feed for 24 h and then the feeder was removed and reweighed to measure consumption (g). Individuals were monitored hourly from the time the feeders were removed until they died from starvation so that survival times between the bees fed the two treatments could be compared.

3.3.6 Data analysis

Survival data were analyzed using Cox regression proportional-hazards models (survival package in R). For honey and bumble bees, we controlled for a colony effect by including a frailty function in the models. Cox models cannot run on completely censored data (because in such a case there are no known endpoints to any of the life spans of the organisms in question), which occurred once in our honeybee data set (treatment 5). We changed the value of one individual in this treatment on the last day of the experiments to “dead” to avoid complete censoring, then modelled the unaltered data with Kaplan-Meier survival analysis with log-rank tests to test the robustness of this method (Tragust et al. 2013). Dose response data for the honeybees were analyzed 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 United States Environmental Protection Agency’s ecological effects test guidelines (USEPA 1996).

For behavioural data, the total proportion of time an individual spent performing each behaviour was calculated. For each behaviour, the control and GTX treatments were
compared using a Mann Whitney U test. For bumble and solitary bee consumption data, the
daily average consumption was calculated for each individual and likewise 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 data and parasite loads for the bumblebee multiple stressors assays
were analyzed using linear mixed effects models, with treatment as a fixed factor and colony
as a random factor (package nlme in R). Parasite load was log transformed in order to meet
the assumptions of normality. All analyses were run in R (version 3.0.2).

3.4 Results

3.4.1 Hypothesis 1: Effects on native bee survival

In the honeybee survival assay, treatment had a significant effect on survival (Cox proportional
hazards model, $\chi^2_4 = 150.8, P < 0.001$, Figure 3.1a.). All solutions containing GTX I (treatments
1, 3 and 4) increased mortality compared with honeybees fed only GTX III (treatment 5) or
controls (treatment 2) (Figure 3.1a). The model revealed that honeybees fed *R. ponticum*
nectar (treatment 1) had a 12 fold increased risk of death (treatment 2, $P < 0.001$). Bees fed
the treatment solutions that contained GTX I (treatments 3 and 4) had a 21 fold increased risk
of death ($P < 0.001$). After correcting for multiple testing, pair-wise log rank tests revealed
there was no significant difference in mortality between honeybees fed *R. ponticum* nectar
(treatment 1) and those fed the artificial nectar + GTX I (treatments 3 and 4, Figure 3.1a).
Honeybees fed the treatment solution containing only GTX III (treatment 5) did not have an
increased risk of mortality ($P = 0.210$). Colony was included in the model as a random effect
but had no impact on survival ($P = 0.920$).

In contrast to the honeybee results, consumption of GTXs did not cause an acute lethal
response in bumblebees. In the seven-day assay (where bumblebees were unrestrained)
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 (Figure
3.1b). In the extended assay comparing the control treatment (treatment 2) and bumblebees
fed GTX I and III (treatment 3), no bumblebees in either treatment died in the 30 day period
(Figure 3.1c.). The 24 h harnessed assay showed similar results; no bumblebees in any of the
five treatments died in the six-hour period after they were fed. Due to the extreme level of censoring, survival analysis was not carried out on these data.

In the 30-day solitary bee assay, there was an initial die-off of 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) had died (Figure 3.1d.). Survival analysis indicated that treatment, however, had no significant effect on survival (likelihood ratio test: $\chi^2 = 0.3$, $P = 0.583$).

Figure 3.1. Survival curves demonstrating the impact of consumption of GTX from R. ponticum nectar on a. restrained honeybees fed treatments 1-5 and observed for a six hour period, b. unrestrained bumblebees fed treatments 1-5 and observed for 7 days, c. unrestrained bumblebees fed treatments 2 and 3 and observed for 30 days and d. unrestrained solitary bees fed treatments 2 and 3 and observed for 30 days. Analysis was carried out using Cox proportional hazards regression models. Multiple log-rank tests were carried out on the honeybee data in order to compare the five treatments, correcting for multiple comparisons between treatment levels using a sequential Bonferroni correction. The bumblebee data were too heavily censored to carry out survival analysis (both for the 7 day and 30 day assay).
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 ecologically-relevant 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 determined to be 0.212 μg/mg for honeybees, approximately half the concentration found in *R. ponticum* nectar (Appendix C, Table C.2). The value for the 48 h LC$_{50}$ was lower still, 0.172 μg/mg (Table C.2).

3.4.2 Hypothesis 2: Sublethal effects

For the bumblebees, treatment did not have a significant effect on any recorded behaviours (Table 3.2). No bumblebee individuals were observed to be paralyzed or exhibit excessive grooming.

For the solitary bees, treatment had a significant effect on two behaviours (Table 3.2). 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 (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 ($W = 228$, $P = 0.006$).

Table 3.2. Comparison of the behaviour of bumblebees and solitary bees fed either a control solution (treatment 2) or a solution containing nectar-relevant concentrations of GTXs (treatment 3). Individuals were observed continuously for 90 seconds, and observations were carried out 11 days throughout the 30 day assay on any individuals that survived to that time point. The total proportion of time bees spent on each behaviour was calculated and compared between the two treatments using a Wilcoxon Rank Sum test. "-" Indicates a behaviour was not performed by the bee species. Behaviours that differed significantly are bolded. * indicates significance at $\alpha = 0.05$ level, ** $\alpha = 0.01$ and *** $\alpha = 0.001$.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Bumblebees</th>
<th>Solitary bees</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test statistic (W) P-value</td>
<td>Test statistic (W) P-value</td>
</tr>
<tr>
<td>Exploring</td>
<td>43.5 0.106</td>
<td>209 0.140</td>
</tr>
<tr>
<td>Still/resting</td>
<td>93.5 0.225</td>
<td>192.5 0.341</td>
</tr>
<tr>
<td>Consumption of treatment solution</td>
<td>61.5 0.561</td>
<td>181.5 0.2707</td>
</tr>
<tr>
<td>Pollen manipulation</td>
<td>66.0 0.359</td>
<td>-</td>
</tr>
<tr>
<td>Grooming</td>
<td>75.5 0.862</td>
<td>140.5 0.458</td>
</tr>
<tr>
<td>Flying</td>
<td>71.0 0.977</td>
<td>228 0.006 **</td>
</tr>
<tr>
<td>Distress behaviours</td>
<td>-</td>
<td>81</td>
</tr>
</tbody>
</table>

52
Bumblebees consumed on average 0.293 ± 0.0198 g solution daily but there was no significant effect of treatment on consumption ($W = 75.0$, $P = 0.887$, Figure 3.2a). Overall, solitary bees consumed less than bumblebees (solitary bee daily mean = 0.0357 g ± 0.002), and there was a significant effect of treatment on solution; solitary bees fed the control solution consumed on average double that of solitary bees fed the GTX solution ($W = 254.5$, $P = 0.011$, Figure 3.2b).

Figure 3.2. Comparison of mean consumption data (± 1 S.E.) for a. bumblebees (n = 12) and b. solitary bees (n= 18) fed a control solution (treatment 2, grey) or a solution containing nectar-relevant concentrations of GTXs (treatment 3, black) 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 calculated, and compared between the two treatments using a Wilcoxon Rank Sum test. ** represents significance at the $\alpha = 0.01$ level.

3.4.3 Hypothesis 3: Additional stressors

Parasite assay

All bumblebees were infected with the parasite at day 12 except for two individuals, which were excluded from the analysis. 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 ($\chi^2 = 0.57, P = 0.508$). Ten days post-infection, the parasite loads of the bees fed the GTX I and III treatment were on average slightly higher than those fed the control treatment (Figure 3.3b, 4777 cells/µL ± 529.5 vs. 4086 cells/µL ± 540.7 respectively), 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 ($F_{1,145} = 1.528, P = 0.220$). Bees fed GTX I and III
were no more likely to be infected with *C. bombi*, nor were their parasite loads significantly different from control bees.

**Survival under stress assay**

Bees fed the control solution (solution 2) consumed slightly less than bees fed the solution containing GTX I and III (solution 3, mean= 0.278 g vs. 0.296 g controlled for evaporation, Figure 3.3d); however, this difference was not significant, nor were there any differences in consumption between colonies (F = 0.349, P = 0.5566). Survival analysis revealed that colony had a significant impact on survival (*P* < 0.001). However, the main variable in question, treatment solution, did not significantly impact bumblebee survival time (Figure 3.3c, \( \chi^2_{18,8} = 29.8, P = 0.210 \)).

![Graphs](image)

Figure 3.3 Results from two experiments investigating combined effects of consumption of GTXs and additional stressors (parasite infection and food stress) on bumblebee workers. 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.
3.5 Discussion

Our study is the first to show that naturally occurring concentrations of nectar secondary compounds in a well-known plant can kill or alter the behavioural response of multiple bee species. Furthermore, this is the first study to our knowledge to investigate how nectar secondary compounds impact an *Andrena* species. The ecological impact of these findings is particularly important from a conservation perspective because our study species, *R. ponticum*, is a severely invasive plant in Western Europe. We demonstrate that invasive *R. ponticum* presents a previously unacknowledged threat to ecologically and economically important native bee species.

3.5.1 Impacts on survival and sublethal effects

While GTX 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 GTXs, and *A. carantonica* exhibited reduced consumption and behavioural indicators of toxicity. Our assays demonstrate that GTX I, but not GTX III, is the toxic component of *R. ponticum* nectar for honeybees. What is remarkable is that 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). Presumably these honeybee subspecies are more tolerant of GTXs than the northern European subspecies *A. mellifera mellifera*, although it is also possible that nectar GTX content may be lower in *R. ponticum* in these areas.

In mammals, GTXs act on the sodium channels of cell membranes in the central nervous system; they bind to voltage-dependent sodium channels in their open state, preventing them from inactivating and leading to hyperpolarisation (Koca and Koca 2007). Species-specific lethality of plant secondary compounds, as we observed in our three bee species, can result when organisms vary in their capacities for performing post-ingestive methods of coping with plant secondary metabolites (Berenbaum 1978, 1981, Ivie et al. 1983). Such methods may include rapid excretion, sequestration of toxins, detoxification and/or target-site insensitivity (Feeny 1992, Slansky 1992). *A. mellifera’s* genome in particular contains only 46 genes coding for cytochrome P450 monooxygenases (a superfamily of enzymes associated with detoxification). This constitutes a reduction of > 50% compared to Dipteran species (Claudianos et al. 2006), indicating a poor capacity for detoxification. Given the mode of action of GTXs, however, it is perhaps more likely that the species-specific toxicity we observed
is due to target-site insensitivity (Slansky 1992); GTX is likely only to impact species when it can act as an activator of sodium channels.

Although nectar secondary compounds have occasionally been shown to cause rapid mortality in honeybees, (reviewed in Pryce-Jones 1942, Majak et al. 1980, Adler 2000), empirical evidence for truly “toxic” nectar is rare. Even sublethal impacts, such as reduced mobility and vigour, are often only observed when concentrations of toxins are artificially high (Cook et al. 2013, Manson et al. 2013). Toxic nectar secondary compounds may deter vital pollinators and therefore decrease plant fitness (Wright et al. 2010, Adler and Irwin 2012, Kessler et al. 2012), thus pollinators may select for concentrations below their thresholds of impact or detection (Wright et al. 2013, Tiedeken et al. 2014).

3.5.2 Additional stressors

Bumblebees are the main visitors and pollinators of *R. ponticum* in its invasive range (Stout et al. 2006), and the differential toxicity of GTXs across our three target taxa may explain this. Interestingly, GTX consumption had no negative synergistic effects when combined with other stressors and no positive impacts on bumblebees challenged by pathogens, in contrast to previous research (Manson et al. 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 *Crithidia bombi*. These assays indicate that even in the presence of additional stressors, *R. ponticum* nectar can provide a useful forage resource for *B. terrestris*.

3.5.3 Impacts of Invasive plants on pollinators

Our study demonstrates that in the case of *R. ponticum*, the impacts of invasion for pollinators vary in both direction and magnitude, and are modulated by nectar toxins. 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. The complex communication used by social insects therefore likely prevents direct honeybee mortality from *R. ponticum* nectar consumption (Afik et al. 2008, Tan et al. 2012). Alternatively, independently foraging solitary bees may be more vulnerable if they do not have the ability to taste toxins in nectar or pollen and hence avoid them. Even if honeybees and susceptible solitary bees readily learn to avoid
toxic \( R. \) ponticum nectar, by replacing native vegetation (Cross 1975) and not providing a palatable alternative nectar resource, \( R. \) ponticum further reduces the amount of food available for \( A. \) mellifera, \( A. \) carantonica and possibly other native bee species. Loss of flower-rich habitat is considered the number one contributor to bee declines (Goulson et al. 2008, Potts et al. 2010a), and our study demonstrates that \( R. \) ponticum invasion decreases floral resource availability for native bees unable to tolerate its nectar toxins.

Alternatively, \( R. \) 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, more \( B. \) lucorum and \( B. \) pascuorum colonies have been found 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 able to utilize the novel forage (Graves and Shapiro 2003, Tepedino et al. 2008).

### 3.5.4 Role of nectar toxins in plant invasion

Secondary compounds may be present in nectar as a pleiotropic consequence of chemical defence in other plant tissues, such as roots or leaves (Adler 2000). Such defences could act as "novel weapons," and could contribute to a plant's success in a non-native environment (Callaway and Ridenour 2004). However, animal-pollinated plants must establish mutualistic relationships with pollinators in non-native environments in order to reproduce and spread (sensu mutualist facilitation, Richardson et al. 2000a). Toxic nectar secondary compounds could therefore present a barrier to invasion for some plants that must be overcome by mutualistic facilitation by an unaffected pollinator. \( R. \) ponticum is well integrated into exotic plant-pollinator networks largely due to pollinating Bombus species (Vilà et al. 2009), that are tolerant of nectar GTXs, but this may not be the case for other non-native species with toxins in their nectar. The extent to which invasive plants contain nectar secondary compounds is unknown (but see Tadmor-Melamed et al. 2004), and requires further investigation.

### 3.5.5 Conclusions

We found that a severely invasive plant, \( R. \) ponticum, presents a previously unacknowledged threat to native honeybees and a native solitary bee, but may be an important forage resource for generalist bumblebees. Little research has investigated the impacts of invasive plant species on native pollinators; likewise, the impacts of nectar secondary compounds on pollinators remain largely unexplored. Our study is the first to address these topics simultaneously, and to test the impacts of a nectar secondary compound on three ecologically
and economically important native bee species using a standard protocol. This work shows that nectar secondary compounds can modulate the impacts of plant invasion for pollinators. Furthermore, we demonstrate that nectar toxins could present an additional barrier to invasion for entomophilous plants that must be overcome by mutualistic facilitation by an unaffected pollinating species.
Chapter 4

Insect-flower interaction network structure is resilient to a temporary pulse of floral resources from invasive Rhododendron ponticum

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4 Insect-flower interaction network structure is resilient to a temporary pulse of floral resources from invasive *Rhododendron ponticum*

4.1 Abstract

Invasive alien plants can compete with native plants for resources, and may ultimately decrease native plant diversity and/or abundance in invaded sites. This could have consequences for native mutualistic interactions, such as pollination. Although invasive plants often become well integrated into native plant-pollinator interaction networks, in temperate climates they usually only flower for part of the season. Unless sufficient alternative plants remain and flower outside this period, whole-season floral resources may be reduced by invasion. We hypothesized that the cessation of flowering of a dominant invasive plant would lead to dramatic, seasonal compositional changes in plant-pollinator communities, resulting in knock-on effects for network structure. We investigated variation in floral resources, flower-visiting insect communities and interaction networks during and after the flowering of invasive *Rhododendron ponticum* in four invaded Irish woodland sites.

Floral resources decreased significantly after *R. ponticum* flowering, but the magnitude of the decrease varied among sites. Neither insect abundance nor richness varied between the two periods (during and after *R. ponticum* flowering), yet insect community composition was distinct, mostly due to a significant reduction in *Bombus* abundance after flowering. During flowering, *R. ponticum* was dominant in insect-flower interaction networks. Despite compositional changes, however, network structural properties remained stable after *R. ponticum* flowering ceased: generality increased, but quantitative connectance, interaction evenness, vulnerability, H_2 and network size did not change. This is likely because after *R. ponticum* flowering, two to three alternative plant species became prominent in networks and insects increased their diet breadth, as indicated by the increase in network-level generality. We conclude that network structure is robust to seasonal changes in floral abundance at sites invaded by introduced, mass-flowering plant species, as long as sufficient alternative floral resources remain throughout the season to support the flower-visiting community.
4.2 Introduction

In light of the variety of threats facing plant and pollinator populations (Kearns et al. 1998), understanding and preserving plant-pollinator interactions has become increasingly important. Recently, some studies have moved beyond a single-species approach and instead utilize the analysis of ecological networks to better understand the structure of entire plant-pollinator communities (Memmott and Waser 2002, Vázquez et al. 2009). This work has identified some common properties of plant-pollinator network structure; for example, networks often display nestedness (they have a core group of generalists that interact with one another, with specialists mostly interacting with a subset of species interacting with generalist species), and asymmetry (specialist plants interact with generalist pollinators, and vice versa) (Bascompte and Jordano 2007, Dormann et al. 2009). It is also widely accepted that plant pollinator interactions are largely generalized; the existence of extreme specialists is rarer than once thought (Waser et al. 1996, Bosch et al. 2009). These properties are thought to increase the stability and robustness of networks (Bascompte et al. 2003, Bascompte et al. 2006), especially when faced with species extinctions (Memmott et al. 2004, Kaiser-Bunbury et al. 2010). Studies of network structure are also used to examine perturbations to networks, such as invasion by introduced plants and pollinators.

Quantitative network studies have demonstrated that invasive plants tend to be well integrated into native plant-pollinator networks through native or invasive generalist flower-visitors that incorporate the alien into their diets (Memmott and Waser 2002, Olesen et al. 2002, Morales and Aizen 2006). Many invasive plants have flowers which are functionally simple, with large nectar rewards (Ghazoul 2002, Stout and Morales 2009). They thus often form strong connections with a large proportion of pollinating species and can receive more visits than co-flowering plants (Lópezaraíza-Mikel et al. 2007, Aizen et al. 2008b, Bartomeus et al. 2008), potentially altering network properties such as the distribution of interactions among species in the community (interaction evenness) (Kaiser-Bunbury et al. 2011). Nevertheless, networks often appear to retain characteristics of robust communities even after invasion by alien plants (Padrón et al. 2009, Vilà et al. 2009, Kaiser-Bunbury et al. 2011).

An inherent limitation to community-level studies investigating impacts of invasive alien plants on native plant-pollinator networks is locating comparable, uninvaded control sites, and ensuring that floral abundance is not a confounding factor between invaded and uninvaded sites (Bartomeus et al. 2008, Stout and Casey 2014). Current studies have dealt with these limitations by surveying areas that exhibit only initial stages of invasion (Bartomeus et al. 2008,

With showy floral displays and copious sugar-rich nectar production, invasive alien plants that occur at high relative abundances could be functionally similar to mass-flowering crop species: they may provide a floral pulse that could be a valuable resource to generalist pollinators (Westphal et al. 2003, Westphal et al. 2009, Diekötter et al. 2010, Stanley et al. 2013). In temporal climates in particular, however, the floral resources provided by many invasive plants are temporary because they tend to flower for a relatively short portion of the overall flowering season. Furthermore, invasive plant species have been shown to decrease native plant abundance and diversity (Martin 1999, Levine et al. 2003), which decreases the chances of a consistent, reliable flower supply throughout the season. No study to date has considered seasonal variation in floral resources in communities invaded by an introduced plant species. If floral resource availability decreases enough after the cessation of flowering of an invasive species, native obligate flower-visiting insects that relied heavily on the invasive plant could be negatively affected, resulting in knock-on effects to network structure. Alternatively, if remaining floral resources are sufficient to sustain the pollinator community, network structure may remain relatively unchanged. In this study, we investigated how floral resources and plant-pollinator community structure change after the flowering period of an abundant invasive plant species.

*Rhododendron ponticum* is a severely invasive alien plant species in north-western Europe. It was introduced to the United Kingdom and subsequently Ireland in the eighteenth century as an ornamental species and as game cover (Cross 1975). An evergreen, perennial shrub, *R. ponticum* invades Irish heaths, bogs and particularly woodlands, where it can alter native plant community composition (Cross 1981). *R. ponticum* presents large floral displays comprised of inflorescences with 9-21 pink-purple zygomorphic flowers (Stout 2007b). These flowers produce a large amount of sugar-rich nectar, making them very attractive to native flower-visitors (Stout et al. 2006, Dietzsch et al. 2011). Studies on the reproductive biology of *R.*
*ponticum* in its invasive range demonstrate that the plant is visited by a variety of insect taxa but pollinated mainly by generalist bumblebee (*Bombus*) species (Stout 2007a). Recent work has shown that invasive *R. ponticum* contains high concentrations of a class of plant secondary compounds, usually associated with defence against herbivory, in its floral nectar (Tiedeken et al. 2014). These secondary compounds (diterpenes known as grayanotoxins) are toxic to some pollinating insect species in the plant's invasive range, including honeybees and some solitary bees (Chapter 3). Because *R. ponticum* nectar is toxic to some flower-visitors, when in flower, this invasive plant may provide a significant floral resource pulse to only part of the flower-visiting community.

Using a quantitative analytical approach, this study aimed to investigate the role of *R. ponticum* in four invaded woodland communities in southeast Ireland, and to determine how the insect community responds to changes in floral resource abundance and composition. We surveyed floral abundance and conducted focal observations of the entire flowering plant community while *R. ponticum* was in flower and again after flowering of the invasive ceased, in order to investigate changes in insect-flower communities during these two distinct time periods. Specifically we tested the following hypotheses: 1.) that floral resource availability decreased at invaded sites after the cessation of *R. ponticum* flowering, 2.) that obligate flower-visiting insect diversity, abundance and visitation rates were higher during vs. after *R. ponticum* flowering, and that insect community composition differed during the two periods, and 3.) that insect-flower interaction network structure (i.e. size, connectance, evenness, weighted plant and animal linkage and level of specialization) changed after the cessation of *R. ponticum* flowering, with smaller and more fragmented networks after *R. ponticum* flowering.
4.3 Materials and Methods

4.3.1 Study sites

Observations of insect-flower interactions were carried out at four native mixed or oak woodland forest sites invaded by *R. ponticum* (Co. Wicklow, southeast Ireland, Table 4.1). In order to standardize abiotic conditions and plant communities, sites were selected that were similar in aspect, elevation and invasion intensity of *R. ponticum* (*R. ponticum* plant cover accounted for approximately one third of the total area of each site, 33.2% ± 8.2% (mean ± SD); coverage estimates were obtained using 20 x 20 m quadrats). Sites were on average 22.33 ± 9.83 km apart to reduce the possible overlap of pollinator communities based on their predicted foraging ranges (Knight et al. 2005a). Because *R. ponticum* requires high light intensity in order to germinate, it often invades forests where there has been a disturbance that causes openings in the canopy (e.g. tree felling), or at edge habitats created by streams or roads (Cross 1981). At our study sites additional flowering species often occurred near these edges as well as in clearings in the forest. Sites were thus defined as 100 x 50 m areas incorporating the portion of the forest invaded by *R. ponticum* as well as any edge habitat that bordered the invaded area.

Study sites were located in forests owned by the state-sponsored private company Coillte, and all necessary permissions were obtained prior to the study. Data on insect and plant surveys are deposited in Ireland’s National Biodiversity Data Centre.

Table 4.1. Characteristics of four study sites in southeast Ireland, County Wicklow, including dominant alternative (non-Rhododendron) plant species during (R1) and after (R2) flowering of invasive *Rhododendron ponticum*.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Altitude (m)</th>
<th>Sampling round</th>
<th>Dominant alternative plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossover</td>
<td>52.894 N</td>
<td>6.400 E</td>
<td>R1</td>
<td><em>Hyacinthoides non-scripta</em>, <em>Ulex europaeus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>165</td>
<td>R2</td>
<td><em>Rubus fruticosus</em>, <em>Galium aparine</em></td>
</tr>
<tr>
<td>Dunran</td>
<td>53.060 N</td>
<td>6.102 E</td>
<td>R1</td>
<td><em>Cytisus scoparius ssp scoparius</em>, <em>Veronica chamaedrys</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>160</td>
<td>R2</td>
<td><em>Stachys sylvatica</em>, <em>Digitalis purpurea</em></td>
</tr>
<tr>
<td>Shankhill</td>
<td>53.192 N</td>
<td>6.427 E</td>
<td>R1</td>
<td><em>Stellaria holostea</em>, <em>V. chamaedrys</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>284</td>
<td>R2</td>
<td><em>D. purpurea</em>, <em>G. aparine</em></td>
</tr>
<tr>
<td>Trooperstown</td>
<td>53.017 N</td>
<td>6.274 E</td>
<td>R1</td>
<td><em>H. non-scripta</em>, <em>V. chamaedrys</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>185</td>
<td>R2</td>
<td><em>S. sylvatica</em>, <em>D. purpurea</em></td>
</tr>
</tbody>
</table>
4.3.2 Plant and insect sampling

In 2011, each site was sampled on at least three distinct days during *R. ponticum* flowering (24 May–28 June, hereafter referred to as **sampling round one**) and again immediately after *R. ponticum* flowering ended (4-26 July, hereafter referred to as **sampling round two**). Communities were sampled using the timed observation method (Morales and Aizen 2006, Olesen et al. 2008, Kaiser-Bunbury et al. 2011). Timed observations help alleviate the bias of overestimating the degree of specialization of rare plants by standardizing observation times (Gibson et al. 2011). Observations of each plant species were made on at least three distinct days during each sampling period and each day of sampling comprised 3 × 10 min observations, (morning (9:00-12:00), midday (12:00-14:30), and afternoon (14:30-17:30)) in order to account for any temporal variation in visitation patterns. Thus we aimed to observe each plant species for a total of 1.5 h/site/sampling period. Inclement weather and differences in flowering phenology reduced the total observation time/species to an average of 1.23 ±0.44 h, however relatively limited sampling effort has been shown to capture a large proportion of the functionally most important community members in plant-pollinator networks (Hegland et al. 2010). Observations were carried out on dry days when the temperature was > 12°C and wind speeds were ≤ 4 according to the Beaufort scale.

During our censuses, we recorded the identity of all diurnal, obligate flower visitors to flowering branches (shrubs and treelets) or flower patches (herbs) (Power and Stout 2011). Although facultative flower visitors (including beetles and some Dipteran species) may play a role in pollination and plant-pollinator network structure, obligate visitors, including bees (Hymenoptera: Apidae), hoverflies (Diptera: Syrphidae), and butterflies (Lepidoptera), are often the most important and effective pollinators of wild and crop plants (Sugiura 1996, Vance et al. 2004, Winfree et al. 2007). In addition, because they rely completely on floral resources for food as adults, they are most likely to be affected by changes in floral abundance and were thus the focus of this study. A visit (synonymous with interaction) was defined as any contact between the flower and the insect. The number of floral units observed during each census and the total number of floral units visited by each insect was noted. A floral unit was defined as a single flower head, or part of a multiple head, from which a medium-sized bee has to fly rather than walk to reach another floral unit of the same species (Dicks et al. 2002).

Where possible, insects were identified on the wing in the field. Unknown specimens were captured and identified to the lowest possible taxonomic category (usually species level).
Bombus lucorum, Bombus cryptarum, Bombus magnus and Bombus terrestris are part of the Bombus sensu stricto species complex, and were thus grouped as “B. lucorum aggregate” because of their morphological similarity (Murray et al. 2008, Carolan et al. 2012); a previous study found that approximately two-thirds of individuals of this aggregate in this area are B. lucorum (Byrne 2010). Bumblebees, hoverflies and butterflies were identified to species level using the appropriate field guides and keys (Stubbs and Falk 1983, National Biodiversity Data National Biodiversity Data Centre 2010), except for certain hoverfly genera that were difficult to distinguish. Melanostoma/Platycheirus species were grouped together because of their morphological similarity, and species of Xylota, Syrphus and Meliscaeva were identified to genus only. Subsequent sampling at the site however demonstrated that the number of species from each of these genera were low, and thus unlikely to affect network structure. An insect reference collection is deposited with the Plant-Insect Interactions research group at Trinity College Dublin. Flowering plant identification followed Parnell and Curtis (2012) and Rose (2006).

We collected floral abundance data at our sites in order to investigate changes in floral resources between the two rounds of sampling, and to weight interactions by the abundance of flowering plant species (Kaiser-Bunbury et al. 2009, Kaiser-Bunbury et al. 2011). Established R. ponticum grows in dense stands that make random quadrat sampling at sites impossible. Instead, our sampling method was a stratified randomized approach, reflecting the relative abundance of R. ponticum at each site (approximately one third cover). Eight 10 m transects were established in areas free from R. ponticum cover, and the number of floral units of each “non-Rhododendron” species was recorded in three 1 x 1m quadrats along each transect (at 0, 5 and 10 m, 24 quadrats). To sample floral abundance in the area covered by R. ponticum, twelve 1 x 1 m quadrats were placed at waist height on twelve randomly selected R. ponticum plants and the number of floral units in each counted. This sampling method was replicated three times throughout each period of sampling at each site at the same time as insect observations were made. The floral abundance of each species was calculated by dividing the total number of flowers by the total number of quadrats sampled at each site (mean number of flowers/m²), and was used in order to weight networks by the relative abundance of each flowering plant species (Kaiser-Bunbury et al. 2011).

We used the total number of observations of each insect species as a measure of abundance of insects at our sites (Bartomeus et al. 2008, Kaiser-Bunbury et al. 2011). In addition to total insect abundance and richness, a number of other parameters were also compared between
sampling rounds including a.) bumblebee abundance, b.) bumblebee richness, c.) hoverfly abundance and d.) hoverfly richness. Solitary bees and butterflies were too rare at sites to be analyzed, but were included in interaction networks (see next section).

4.3.3 Flower-visitor networks

We constructed two fully quantitative interaction matrices for each site, one for each round of sampling. Following the methodology of Kaiser-Bunbury et al. (2011), we used mean interaction frequencies in our data matrices to account for slight differences in sampling effort between plant species at a site. We used interaction frequencies to represent interaction strength between plant and insect species, and quantified visits based on the floral abundance of the interaction partner; 'mean interaction frequency' was represented as the total number of visits /flower/hour of animal species $a$ to plant species $p$ multiplied by the floral abundance (average floral units/ m$^2$) of plant species $p$ (Vázquez et al. 2005, Bascompte et al. 2006, Vázquez et al. 2007, Kaiser-Bunbury et al. 2011). Due to the small size of our daily networks, data from each of the three visits to a site were combined and networks and network parameters were calculated at the site level for each sampling period (Stout and Casey 2014). Mean interaction frequencies of flower visitors at each site were also used to construct non-metric multi-dimensional scaling (nMDS) plots in order to investigate patterns of flower-visiting insect communities.

For comparison with other networks, we calculated qualitative network parameters for our sites during each sampling round (Table 4.2) following Dorman et al. (2009). We also calculated quantitative network descriptors in order to compare the structure of the insect-flower interaction networks between the two sampling periods. Quantitative as opposed to qualitative parameters incorporate the interaction frequency of individual species and are preferable because they are more robust than their qualitative equivalents to variations in sampling effort and changes in network size (Bersier et al. 2002, Tylianakis et al. 2007). Using the "networklevel" command in the bipartite package (Dormann et al. 2008) in R, version 3.0.2 (R Development Core Team 2011), we calculated: 1. **Quantitative connectance** (the
realized proportion of all possible links weighted by the quantitative visitation rate of each species, (Bersier et al. 2002, Kaiser-Bunbury et al. 2011), calculated as linkage density/species richness (P+A)); 2. Interaction evenness (a measure of how well distributed interactions are among species within communities, based on the Shannon index and calculated as IE=\(p_{pa}\log_2 p_{pa}/\log_2 S\), where S= total number of insect-flower interactions in the network and \(p_{pa}\) is the proportion of interactions between plant \(p\) and animal \(a\) (Tylianakis et al. 2007, Kaiser-Bunbury et al. 2011)); 3. Generality (or the weighted linkage for insect visitors, used to represent the level of generalization in the diets of pollinators, a.k.a. diet breadth (Memmott et al. 2004), and calculated as the weighted mean number of plant species per visitor species); 4. Vulnerability (or the weighted linkage for plants, calculated as the weighted mean number of insect visitor taxa per plant species (Kaiser-Bunbury et al. 2011), a.k.a. pollinator breadth); and 5. \(H^2\) (a measure of the overall level of specialization in a network, ranges between 0 (no specialization) and 1 (perfect specialization), calculated based on the difference between realized and expected interactions (Dormann et al. 2009)).

4.3.4 Data analysis

Network parameters and insect and taxon-specific abundance, visitation and richness, were calculated as mean values per sampling round, averaged across all four sites; thus, they were compared between the two sampling rounds using univariate analyses (paired t-tests). The limited power associated with our low sample size (n = 4 networks per sampling round) is largely justified by the considerable effort involved in sampling entire insect-flower interaction communities in a limited time period (during the flowering period of the invasive species), and is not unusual for similar studies (Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2008).

Floral abundance data were analyzed using mixed effects models in SPSS (response variable = floral units per meter^2). Sampling round (during or after flowering), site (1-4), and their interaction were included in the model as fixed factors, and quadrat nested within site was included as a random factor. In order to investigate how \(R. ponticum\) influences floral resources available to obligate flower visitors, two separate models were run; one for the total floral units recorded (complete model), and one for only non-\(Rhododendron\) floral units. Models were validated by plotting standardised residuals against fitted values, and floral abundance was log +1 transformed where necessary. Fisher LSD post hoc comparisons were used to compare floral abundance at each site during the two sampling rounds.
Differences in the composition of available floral resources between the two rounds of sampling were visualised using non-metric multi-dimensional scaling (nMDS) plots based on Bray-Curtis dissimilarity matrices in PRIMER 6 (Version 6.1.13) (Plymouth Routines in Multivariate Ecological Research, Plymouth Marine Laboratory, Plymouth, UK). Floral unit data were square root transformed in order to prevent highly abundant plant species (i.e. *R. ponticum*) from dominating the analyses. We tested for differences in floral composition between the two sampling rounds using non-parametric multivariate analysis of variance (Permutational MANOVA, “PERMANOVA”), with sampling round included as a fixed factor and site as a random factor. The PRIMER routine SIMPER (Similarity of Percentages) analysis was used to identify which species were important in distinguishing among communities from the different rounds of sampling. SIMPER tables (see Appendix D) included species until a cumulative 70% of dissimilarity was accounted for. The same multivariate techniques and model design used for the floral abundance data were employed in order to investigate differences in patterns of mean insect visitation frequencies.

4.4 Results

In total, 1,446 insect-flower interactions were observed in approximately 108 h of focal observations during the two rounds of sampling. Of those visits, 675 and 771 were observed during and after *R. ponticum* flowering respectively. Floral visitors were comprised of insects from three Orders; Diptera (the most species-rich group, 16 Syrphid taxa), Hymenoptera (five bumblebee species, one honeybee species and two solitary bee genera), and Lepidoptera (one butterfly and one moth species) (Appendix D). The Syrphids accounted for the majority of overall interactions at our sites (78.8%), followed by the bees (20.2%) and butterflies and moths (1.6%). All observed insect taxa were native to Ireland.

4.4.1- Changes in floral abundance

A total of 35 plant species were observed during the two sampling rounds, 25 species during *R. ponticum* flowering and 20 species after flowering ceased, with 10 species flowering during both rounds. Plant species richness at sites did not vary significantly between the two sampling rounds (paired t-test: t = 0.3, d.f. = 3, P = 0.79). During *R. ponticum* flowering, *Hyacinthoides non-scripta* and *Cytisus scoparius ssp. scoparius* were often the most abundant non-*Rhododendron* flowers, although their mean floral abundance per m² was 2-10 times less than that of *R. ponticum*’s. In the second round of sampling, the average floral abundance per
m² of all flowering plant species was low in comparison to *R. ponticum* in the first round, but *Rubus fruticosus*, *Stachys sylvatica* and *Digitalis purpurea* flowers were often the most abundant species (Table 4.1). While *R. ponticum* was in flower, (sampling round 1) it comprised on average just over two-thirds of the total available floral units (average 67.37% ± 13.7, Figure 4.1).

Overall, mean floral units per m² decreased significantly after *R. ponticum* stopped flowering (*F*₁,₇₁₆ = 30.363, *P* < 0.001, Figure 4.1). The magnitude of this decrease, however, depended on the site being sampled (site*sampling round interaction, *F*₃,₇₁₆ = 2.939, *P* = 0.033). Post hoc comparisons using the Fisher LSD test revealed that there was a significant decrease in floral units per m² at all sites (Crossover: *P* < 0.001, Shankhill: *P* = 0.024, Trooperstown: *P* < 0.001) except for Dunran (*P* = 0.586). There was no consistent pattern in abundance of non-*Rhododendron* flowering units among sites between rounds (site*sampling round interaction, *F*₃,₄₇₆ = 4.095, *P* = 0.007): Fisher LSD post hoc comparisons revealed a decrease in non-*Rhododendron* floral units at Crossover (*P* = 0.050), an increase at Dunran (*P* = 0.014) and Shankhill (*P* = 0.037), and no significant change at Trooperstown (*P* = 0.755) (Figure 4.1).

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**Figure 4.1** Total number of available floral units, comprised of invasive *R. ponticum* (light grey bars) and alternative plant species (dark grey bars), during the flowering of *R. ponticum* (R1) and after cessation of the flowering of the invasive species (R2) at four invaded Irish woodland sites.
Multivariate analysis showed the composition of the floral resources available to obligate flower visitors during round one (when *R. ponticum* was in flower) was significantly different from that of round two (after flowering ceased, main effect sampling round: $F_{1,3} = 10.58, P = 0.027$). This difference was of course mostly attributed to the cessation of *R. ponticum* flowering, but also to the start of flowering of a few abundant alternative plant species, namely *Stachys sylvatica*, *Digitalis purpurea* and *Rubus fruticosus* (Figure 4.2, Appendix D, Table D.2). The model also revealed a significant main effect of site ($F_{3,16} = 19.29, P = 0.001$) and a significant site*sampling round interaction ($F_{3,16} = 9.70, P = 0.001$); flowering communities were more distinct between sites after *R. ponticum* flowering, and more similar during flowering.

![Non-metric Multi-Dimensional Scaling plot of floral abundance data for each site.](image)

**Figure 4.2** Non-metric Multi-Dimensional Scaling plot of floral abundance data for each site (each point on the graph represents a replicate of floral abundance sampling, n=3 replicates per site per sampling round). The closer the points, the more similar the identity and abundance of flowering plant species. Light grey squares represent sites sampled during *R. ponticum* flowering and black circles represent the same sites after flowering of the invasive species ceased. Label codes indicate the sampling round (1 or 2), the site name (C = Crossover, D = Dunran, S = Shankhill, T = Trooperstown), and the replicate (1, 2, or 3). Data were square root transformed to balance contributions of rarer and dominant flowering species.
4.4.2- Changes in flower-visitor diversity and visitation

Overall, insect species richness (ISR) and total insect visits (TIV) at sites did not differ between the two sampling rounds (ISR: t = 0.200, TIV: t = 0.777, d.f. = 3, P > 0.05, Figure 4.3a & b). The total visits observed to non-*Rhododendron* plant species however, increased significantly after the cessation of *R. ponticum* flowering (t = 3.674, d.f. = 3, P = 0.0349, Figure 4.3d). Insect species richness to non-*Rhododendron* flowering plants was not significantly different between the two sampling rounds (t = 2.376, d.f. = 3, P = 0.098, Figure 4.3c). The number of visits observed from bumblebees decreased at our sites after the cessation of *R. ponticum* flowering (t = 3.449, P = 0.041); however, bumblebee and hoverfly species richness and observed hoverfly visits did not change significantly (bumblebee richness: t = 1.732, hoverfly richness: t = 1.058, hoverfly visits: t = 1.657, d.f. = 3, P > 0.05, Figure 4.3a & b).

![Figure 4.3](image)

**Figure 4.3** Flower visitor species richness and abundance during *R. ponticum* flowering (round 1, light grey bars) and after the cessation of flowering of the invasive (round 2, dark grey bars). Comparison between round 1 and 2 of a.) site level species richness and b.) number of visits of total insects, bumblebees and hoverflies, c.) insect species richness to non-Rhodo plants and d.) number of visits to non-Rhodo plants. Plots show mean values per round, averaged across the sites, and vertical bars show the standard error of each mean. Number of visits represents the number of floral units visited by insects, however insect abundance (number of individuals, not shown) followed the same patterns. Significant differences in the above parameters when comparing the two rounds of sampling are indicated by * (paired t-test, P < 0.05).
Multivariate analysis revealed that the mean interaction frequencies of insect communities observed during sampling round one (when *R. ponticum* was in flower) were significantly different from those observed in sampling round two (*F*₁,₃ = 4.538, *P* = 0.034, Figure 4.4). The main contributors to this difference were the bumblebees and hoverflies in the genera *Meliscaeva* and *Sphegina*; the mean interaction frequency of *B. lucorum agg.* and *Sphegina clunipes* decreased once *R. ponticum* flowering ceased, while that of *Meliscaeva* increased (Appendix D, Table D.3).

**Figure 4.4** Non-metric Multi-Dimensional Scaling plot of flower-visitor interaction frequencies at sites invaded by *R. ponticum* during (round 1) and after (round 2) flowering. Site level mean interaction frequencies of pollinator groups were used to calculate the resemblance matrix. The closer the points, the more similar the identity and abundance of interaction frequencies of flower-visitors recorded. Data were square root transformed to balance contributions of rarer and dominant insect visitors.

### 4.4.3- Changes in insect-flower interaction networks

Networks from both sampling rounds were small (minimum of nine plant species and 13 animal species, maximum of 13 plant species and 19 animal species, Table 4.2), but network
size did not differ significantly between the two sampling rounds (paired t-test, \( t = 0.480, \text{d.f.} = 3, P = 0.664 \)).

During its flowering period, *R. ponticum* was well integrated into and dominant in insect-flower interaction networks at our sites (Figure 4.5 a, c, e & g). It was well integrated because it was the plant with the highest linkage in all four sites (Table 4.2), interacting with on average 74.10% (± 13.1) of insect species. Bumblebees were the most common visitors to *R. ponticum*, but visits from hoverflies were also common. *R. ponticum* also dominated the networks in terms of visitation: 55.65% ± 8.04 of all interactions were to *R. ponticum*. None of the round one networks exhibited significant compartmentalization, further demonstrating that the invasive species was well integrated (Figure 4.5 a, c, e & g), and the majority (average 75.23% of species ±16.6) of insect species interacting with *R. ponticum* also interacted with at least one additional plant species.

Of the quantitative network parameters calculated for each site, only generality differed between the two sampling rounds, increasing significantly after *R. ponticum* stopped flowering (\( t = -3.516, \text{d.f.} = 3, P =0.039 \), Figure 4.6c, Figure 4.5). In contrast, quantitative connectance, interaction evenness, vulnerability and \( H^2 \) did not change significantly after *R. ponticum* stopped flowering (QC: \( t = 0.123 \), IE: \( t= -2.251 \), V: \( t = 1.060 \), \( H^2 \): \( t= 0.457 \), d.f. = 3, \( P >0.05 \); Figure 4.6a-b, d-e).
Table 4.2. Qualitative network parameters for insect-flower interaction networks during (R1) and after (R2) flowering of invasive *R. ponticum*. Qualitative network parameters include the number of plant species (P), number of flower-visiting insect species (A), the total number of unique flower-insect interactions (links, L), the total number of interactions between plants and insects (interactions, I), ratio of animal to plant species (A/P), full network size (S=A*P), qualitative connectance (C=100* L/S) and mean and maximum plant and animal linkage.* indicates that *R. ponticum* was the plant species with the highest linkage in the network.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of plant taxa (P)</th>
<th>Number of insect taxa (A)</th>
<th>Number of links (L)</th>
<th>Number of visits (V)</th>
<th>Ratio (A/P)</th>
<th>Network size (S)</th>
<th>Connectance (C)</th>
<th>Maximal plant linkage (L_max)</th>
<th>Maximal animal linkage (L_max)</th>
<th>Mean plant linkage (L_p)</th>
<th>Mean animal linkage (L_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossover</td>
<td>R1 10</td>
<td>16</td>
<td>53</td>
<td>180</td>
<td>1.60</td>
<td>160</td>
<td>313.13</td>
<td>11*</td>
<td>9</td>
<td>5.30 ± 3.40</td>
<td>3.31 ± 2.68</td>
</tr>
<tr>
<td></td>
<td>R2 11</td>
<td>17</td>
<td>66</td>
<td>299</td>
<td>1.55</td>
<td>187</td>
<td>35.29</td>
<td>11</td>
<td>10</td>
<td>6.00 ± 2.82</td>
<td>3.88 ± 3.25</td>
</tr>
<tr>
<td>Dunran</td>
<td>R1 13</td>
<td>15</td>
<td>54</td>
<td>179</td>
<td>1.15</td>
<td>195</td>
<td>27.69</td>
<td>12*</td>
<td>10</td>
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<tr>
<td></td>
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<td>15</td>
<td>45</td>
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<td>1.36</td>
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<td>27.27</td>
<td>9</td>
<td>10</td>
<td>4.09 ± 3.01</td>
<td>3.00 ± 2.62</td>
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<td>200</td>
<td>1.90</td>
<td>190</td>
<td>27.37</td>
<td>14*</td>
<td>10</td>
<td>5.20 ± 3.61</td>
<td>2.74 ± 2.70</td>
</tr>
<tr>
<td></td>
<td>R2 12</td>
<td>16</td>
<td>59</td>
<td>178</td>
<td>1.33</td>
<td>192</td>
<td>30.73</td>
<td>13</td>
<td>10</td>
<td>4.92 ± 3.34</td>
<td>3.69 ± 2.85</td>
</tr>
<tr>
<td>Trooperstown</td>
<td>R1 9</td>
<td>13</td>
<td>30</td>
<td>116</td>
<td>1.44</td>
<td>117</td>
<td>25.64</td>
<td>8*</td>
<td>8</td>
<td>3.33 ± 2.12</td>
<td>2.31 ± 2.25</td>
</tr>
<tr>
<td></td>
<td>R2 9</td>
<td>16</td>
<td>43</td>
<td>99</td>
<td>1.78</td>
<td>144</td>
<td>29.86</td>
<td>10</td>
<td>8</td>
<td>4.78 ± 3.23</td>
<td>2.69 ± 2.06</td>
</tr>
</tbody>
</table>
Figure 4.5 Quantitative insect-flower interaction networks for four Irish woodland sites during (a, c, e, g) and after (b, d, f, h) *R. ponticum* flowering. For each web, upper bar widths represent pollinator guild abundance while lower bar widths are determined by the interaction strength with insect species. On the lower bars, the number 1 corresponds to *R. ponticum* and is circled in gray; remaining species codes and pollinator guild abbreviations are listed in Appendix D, Table D.1 a-b. Linkage width indicates the frequency of the interaction. One network was calculated for each site during each round of sampling.
Figure 4.6 Comparison of quantitative network parameters calculated from insect-flower interaction networks created by sampling four invaded Irish woodland insect-communities during (light gray) and after (dark gray) flowering of *R. ponticum*. Network parameters analyzed include a.) quantitative connectance, b.) interaction evenness, c.) generality, d.) vulnerability and e.) $H^2$. * indicates a significant difference between the two rounds of sampling (paired t-test, $P < 0.05$). Plots show mean parameters per sampling round across all four sites, and vertical bars show the standard error of each mean.
4.5 Discussion

When in flower, *R. ponticum* is both well integrated and dominant in native insect-flower interaction networks. Our study, however, demonstrates that despite changes in the composition of communities after *R. ponticum* stops flowering, moderately invaded sites contain sufficient remaining floral resources to maintain robust insect-flower interaction network structure.

4.5.1- Changes in floral abundance

As predicted, sites experienced a significant decrease in overall floral abundance after *R. ponticum* stopped flowering. After *R. ponticum* flowering, the abundance and diversity of plant species that remain or come into flower next dictate the severity of the impact of this decline in total floral abundance. Invasive plants often compete with native plants and change plant species composition, resulting in a decrease in plant diversity and abundance (Cross 1981, Pyšek and Pyšek 1995, Martin 1999). Thus, the abundance of flowering species in invaded locations could be low. Surprisingly, our study demonstrates that this is not always the case. There can be significant variation in alternative floral resource abundance among sites, even when the level of invasion is consistent. Dunran and Shankhill both had an increase in alternative (non-*Rhododendron*) floral units after *R. ponticum* flowering ceased, Trooperstown experienced no significant change, and Crossover saw an overall decrease in alternative floral units. Crossover, however, had a much higher number of alternative floral units during *R. ponticum* flowering in comparison to the other sites; thus, although the decrease in round 2 was significant, the overall floral availability was still comparable to the other sites. While our sites were representative of invaded woodlands on the east coast of Ireland, it should be noted that *R. ponticum* cover in woodlands in the west and other habitat types (bogs, heathland) can be substantially higher (Usher et al. 1986, Dietzsch et al. 2011, Stout and Casey 2014). A more consistent and severe decrease in non-*Rhododendron* floral resources may be expected at these heavily invaded sites. Our study is the first to measure seasonal fluctuation in floral resources at sites invaded by an introduced, mass-flowering plant species, and to consider how these fluctuations may directly impact obligate flower visitors.

4.5.2- Changes in flower-visitor diversity, visitation and composition

Total insect abundance and richness at our sites did not change significantly between the two sampling periods, however the number of visits to co-flowering, non-*Rhododendron* species increased significantly when *R. ponticum* was no longer in flower. The majority of studies...
investigating the impact of invasive alien plants on native co-flowering plant pollination find primarily negative effects (Bjerknes et al. 2007, Morales and Traveset 2009). Our findings suggest that negative impacts on the pollination of co-flowering plants may not persist throughout the flowering season, however, further studies investigating pollen deposition and seed set are required to test this hypothesis.

Even though total insect abundance and species richness did not change between the two sampling rounds, the composition of the insect communities visiting flowers was distinct, largely due to a decrease in bumblebee visitation after the cessation of *R. ponticum* flowering. In our networks, the links between bumblebees and *R. ponticum* were strong during sampling round one. Bumblebee richness remained similarly low (5 species) in the second sampling period, however the abundance and visitation of bumblebees at the sites dropped drastically, indicating that *R. ponticum* is an important forage resource for bumblebees (Stout et al. 2006, Dietzsch 2009). The change in the composition of the insect communities after *R. ponticum* stopped flowering may simply have been due to seasonal variation in the abundance or activity of different insect species. However long-season, generalist bumblebees could have left the sites after *R. ponticum* stopped flowering to find more rewarding or abundant forage sources elsewhere (Goulson 2000). Bumblebees are efficient foragers, have large foraging ranges (Knight et al. 2005a), and are able to utilize resources distributed across a landscape scale (Knight et al. 2009). Other insects, such as hoverflies, may not be capable of such long-range foraging.

4.5.3- Changes in insect-flower interaction networks

During its flowering period, *R. ponticum* was well integrated into native insect-flower interaction networks and dominated network structure. It received, on average, half of the overall insect visits at sites, and was by far the most highly connected plant species. This finding is consistent with previous investigations of communities invaded by introduced plant species; for example, three other invasive plants with large showy floral displays, *Impatiens glandulifera, Carpobrotus affine acinaciformis* and *Opuntia stricta*, were also well integrated into network structure, to the point where they received significantly more pollinator visits or higher visitation rates than co-flowering native species (Bartomeus et al. 2008, Bartomeus et al. 2010). To our knowledge however, none of these invasive plants expressed traits that made their rewards unavailable to a large proportion of members of the pollinator community. Even the concealed nectar of *I. glandulifera* is exploited by a wide range of insects (Lopezaraiza-Mikel et al. 2007). *R. ponticum* nectar, in contrast, is toxic to honeybees and at
least one solitary bee species (genus *Andrena*) in its invasive range (Chapter 3). Generalist honeybees are often frequent visitors of invasive plant species, and can significantly alter network structure (Kaiser-Bunbury et al. 2011). The absence of honeybees from *R. ponticum* invaded networks, presumably due to the toxic effects of *R. ponticum* nectar, could therefore impact species interactions and levels of connectance between community members. Regardless of its toxic nectar, however, *R. ponticum* still acted as a super generalist species in our networks (Aizen et al. 2008b, Bartomeus et al. 2008); it interacted strongly with the majority of insect species at our sites.

Despite the decrease in floral resources and the compositional changes to the community, our results demonstrate that network structure remained stable after *R. ponticum* finished flowering. Only generality, or the weighted mean number of plant species per insect species, changed significantly between sampling rounds; it increased after *R. ponticum* stopped flowering. This is probably because no single alternative species replaced *R. ponticum* in terms of dominance of the network. Instead two to three plant species became more prominent in networks. Flower-visitors therefore included more plant species in their diets after the flowering of the invasive species, presumably to obtain sufficient floral resources.

Studies have shown decreases in network size, and visitor species richness and abundance when invasive flowers are removed from invaded sites (Lopezaraiza-Mikel et al. 2007), and differences in interaction evenness (the distribution of interactions between different species in the network) among sites varying in invasion intensity (Kaiser-Bunbury et al. 2011). Furthermore, models which have simulated species removal in order to investigate the impact of species loss on network structure have demonstrated that loss of highly connected community members leads to collapse quicker than loss of less connected species (Memmott et al. 2004, Albrecht et al. 2014). We therefore hypothesized that the structure of invaded networks would change once abundant, dominant *R. ponticum* stopped flowering. On the contrary, network structure remained relatively stable, probably because there was the opportunity for the insect and floral communities to respond to compositional changes (rewiring, Knight et al. 2005b, Burkle and Alarcón 2011), which was not the case in Memmott et al. (2004). Similar to our findings, several recent studies of temporal variation in uninvaded plant-pollinator communities have shown that although the composition of communities changes within and between seasons, network structural properties remain relatively consistent due to re-wiring (Alarcón et al. 2008, Olesen et al. 2008, Petanidou et al. 2008, Dupont et al. 2009). The temporal variation exhibited by our networks was therefore similar
to that of uninvaded networks, regardless of the floral resource pulse provided by *R. ponticum*. This may not be the case at more heavily invaded sites (e.g. Stout and Casey 2014), where native plant diversity could be severely depleted and therefore unable to sustain the insect community after the flowering of the invasive species.

Our results may also be useful from a conservation perspective. Invasive alien plant species are often cleared in order to benefit biological diversity and allow the recovery of ecosystems (Clout and Veitch 2002). If invasive plants integrate into networks and strongly interact with flower-visiting species, their removal could have important and potential detrimental effects on the pollinator community that relied on the invasive as a floral resource, particularly if native flowering plants are not restored (Ferrero et al. 2013). Our results indicate that at least for moderately invaded sites, if *R. ponticum* was removed for conservation purposes, network structure may be resilient to the loss of this highly connected invasive plant.

### 4.5.4 Conclusions

Our findings demonstrate that an entomophilous invasive alien plant can integrate into native insect-flower interaction networks, even when the floral rewards it provides are not suitable for the entire flower-visiting community. Our work also demonstrates that although the composition of flowering plant and insect communities changes at sites after an abundant invasive plant species stops flowering, community structure can remain relatively stable if the flower-visitor community expands its diet and utilizes available alternative floral resources. We conclude that the seasonal impacts of invasion by alien plants on insect-flower interaction networks are dependent not only on the traits of the invasive species but the composition of the native plant community.
Chapter 5

A community-level biogeographical approach to the study of plant invasions: Insect-flower interaction networks in the introduced and native range of *Rhododendron ponticum*

A community-level biogeographical approach to the study of plant invasions: Insect-flower interaction networks in the introduced and native range of *Rhododendron ponticum*

5.1 Abstract

Although biological invasions are by nature biogeographical phenomena, most studies on invasive plants are carried out exclusively in the introduced range. Comparative studies of plant-pollinator communities in both the introduced and native ranges of invasive species could aid in understanding how novel species introductions effect native plant-pollinator community structure. On the one hand, invasive plants may dominate communities to a lesser extent in their native range where co-flowering species have coevolved with the focal species. Alternatively, it is possible that invasive plant species dominate network structure in both ranges due to the attractive and generalist nature of their floral displays. Using *Rhododendron ponticum* as a model species, we present the first comparison of field replicated, quantitative, insect-flower interaction networks in the native and introduced range of an invasive plant species.

Insect flower interaction network structure, as measured by weighted connectance, interaction evenness, vulnerability and $H^2$, was similar for communities in both the native and introduced range. *R. ponticum* acted as a “super generalist” in networks in both ranges; it had the highest species strength of any plant and was strongly connected to most insect functional groups in all eight networks. As predicted by the enemy release hypothesis, visitation from nectar and pollen thieves and evidence of nectar robbing was more frequent on *R. ponticum* individuals in the native range. Our results indicate that invasive plants may alter insect-flower interaction network structure in invaded communities not just because they are novel introductions to the community, but because of the attractive and generalist nature of their floral displays. Our study demonstrates that a comparative biogeographical approach is useful in interpreting the impacts of invasive species on native plant-pollinator communities.
5.2 Introduction

Invasive alien plants are important drivers of global environmental change that can affect the abiotic conditions of environments, biodiversity and ecosystem processes (Sala et al. 2000, Millennium Ecosystem Assessment 2005, Pimentel et al. 2005). In their native range, invasive plant populations are regulated by local conditions; however, when introduced to a new location by humans, invasive plants are exposed to potentially different abiotic and biotic conditions which can impact their abundance and their ecology in general (Mitchell et al. 2006). Invasion is thus a biogeographical phenomenon, yet the majority of studies investigating invasive plant species are carried out exclusively in the introduced range (Richardson et al. 2000a, Hierro et al. 2005).

A comparative biogeographical approach can be useful—and sometimes even necessary—to the field of invasion ecology. For example, comparative studies are needed to determine how often invasive species are actually more abundant in their introduced than their native ranges (Hierro et al. 2005). In addition, hypotheses explaining the success of invasive alien species, including enemy release (Colautti et al. 2004), the evolution of increased competitive ability (Blossey and Notzold 1995), empty niche (MacArthur 1970), and novel weapons (Callaway and Ridenour 2004), assume the pressures controlling populations of invasive species are different between the native and invasive range, and thus require a biogeographical comparison (Hierro et al. 2005). Finally, one of the main goals of invasion ecology is to understand how and why invasive species become integrated into native communities (Davis 2009), for example, through the establishment of mutualistic relationships in the introduced range (Richardson et al. 2000a). Studies of the relationships between invasive plants and mutualists in both ranges can shed light on this important topic.

Many of the existing biogeographical studies of plant invasions are demographic comparisons of the focal species in its native and introduced range (Woodburn et al. 1996, Paynter et al. 2003, Jakobs et al. 2004). Biogeographical studies that focus on biotic interactions, however, are less common. These types of studies can be grouped into one of two categories, studies of antagonistic interactions and studies of mutualistic interactions. The former often test the widely cited enemy release hypothesis, which states that exotics in introduced ranges are more successful because they are released from specialist natural enemies that controlled their populations in native ranges (Mitchell et al. 2006). Comparisons of the prevalence and contribution of folivores and diseases to plant population control in both ranges have been carried out to test this hypothesis (e.g. Castells et al. 2013, Oduor et al. 2013, Blaisdell and Roy
2014), but floral larcenists, another class of plant enemies (Inouye 1980, Irwin and Brody 1999, 2000, Castro et al. 2008), have yet to be studied in this context. Biogeographical studies of mutualistic relationships are less common, and mainly focus on the pollination ecology of invasive plants (Stout et al. 2006, but see Moora et al. 2011, Ollerton et al. 2012b, Montero-Castaño et al. 2014). Entomophilous exotic plants must establish mutualistic relationships with pollinators in the introduced range in order to reproduce and spread (mutualist facilitation, sensu Richardson et al. 2000a). A biogeographical approach has been utilized to compare the pollination ecology and reproductive success of plants in both ranges, in order to disentangle the processes that enable exotics to succeed in new environments (Stout et al. 2006, Montero-Castaño et al. 2014).

Invasive plants can alter both native plant community composition (Cross 1981, Pyšek and Pyšek 1995, Martin 1999, Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2008, Kaiser-Bunbury et al. 2011) and pollinator populations (Tepedino et al. 2008, Moroń et al. 2009); they thus have the potential to impact the structure of entire plant-pollinator mutualistic networks (Memmott and Waser 2002, Olesen et al. 2002). Studies of plant-pollinator network structure have been used to examine perturbations to networks, including invasion by introduced species (Memmott and Waser 2002, Olesen et al. 2002). No study to date, however, has utilized a biogeographical approach in order to better understand the impacts of invasive plants on entire plant-pollinator networks.

Quantitative network studies carried out in introduced ranges have found that invasive plants are often well integrated into native plant-pollinator networks (Memmott and Waser 2002, Morales and Aizen 2006), and can impact network level properties such as the distribution of interactions among species in the community (interaction evenness) (Kaiser-Bunbury et al. 2011). In their introduced range, invasive plants tend to form strong connections with a large proportion of pollinating species (Chapter 4), and can even act as super generalists, receiving more visits than co-flowering native plant species (Lopezaraiza-Mikel et al. 2007, Aizen et al. 2008b, Bartomeus et al. 2008). No study, however, has asked if these characteristics are consistent between an invasive plants’ native and introduced range. Invasive plants may dominate network structure to a lesser extent in their native range, where co-flowering species have coevolved with the focal species and are thus potentially better at competing with it for pollinators. Alternatively, it is possible that invasive plants dominate network structure in both ranges, and that this tendency could help explain the success of invasive plants in introduced ranges. A biogeographical approach could help to address these
questions and improve our understanding of the impacts of plant invasion on plant-pollinator network structure (Table 5.1).

Table 5.1 Examples of questions that could be addressed using comparative biogeographical studies of plant pollinator interaction networks.

<table>
<thead>
<tr>
<th>Questions</th>
<th>Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do plant-pollinator networks in the introduced vs. native range of a plant have similar structural properties and stability?</td>
<td>Calculation and comparison of network level parameters associated with stability (connectance, interaction evenness, web asymmetry, nestedness)</td>
</tr>
<tr>
<td>Is the invasive species equally specialized and connected in the introduced and native range?</td>
<td>Calculation and comparison of species level parameters of the focal invasive species (species degree, strength, partner diversity, number of effective partners)</td>
</tr>
<tr>
<td>Are pollinators from the same guild/families playing the same functional roles in the introduced and native range?</td>
<td>Calculation and comparison of species level parameters of pollinator guilds (species degree, strength, partner diversity, number of effective partners, specialisation)</td>
</tr>
<tr>
<td>Do invasive plant species act as super generalists in both the introduced and native range?</td>
<td>Comparison of plant-pollinator network parameters (such as interaction evenness) and species level parameters and visitation rates to the focal invasive species</td>
</tr>
<tr>
<td>Which visitors integrate invasive plants into plant pollinator networks in the introduced range, and are they closely related to its visitors in the native range?</td>
<td>Comparison of the diversity and abundance of pollinator species visiting the invasive plant in both ranges</td>
</tr>
<tr>
<td>Do co-flowering plants in the native range receive more visits/higher visitation rates than co-flowering plants in the introduced range?</td>
<td>Compare abundance and richness of visitors to native plant species in the introduced and native ranges</td>
</tr>
<tr>
<td>Is the abundance of the invasive species responsible for its dominant role in plant-pollinator networks in the introduced range?</td>
<td>Compare network parameters from sites in the native and introduced range that have an increasing gradient of abundance of the invasive species. Do network parameters follow the same trends in both ranges?</td>
</tr>
<tr>
<td>Do successful entomophilous invasive plant species tend to be highly connected generalists in their native range? Can this pattern help us predict which species may have most success in introduced environments?</td>
<td>Answer using meta-analysis after several biogeographical comparisons of plant species in their native and introduced range have been analyzed using quantitative networks</td>
</tr>
</tbody>
</table>
Using *Rhododendron ponticum* L. as a model species, we present the first biogeographical comparison of field replicated, insect-flower interaction networks in the native and introduced range of an invasive plant. By investigating the entire community of flower-visitors to *R. ponticum*, including both mutualist pollinators and floral larcenists, our community-level approach allows us to test for evidence of enemy release from floral antagonists. Specifically we tested three novel hypotheses:

**Hypothesis 1:** Networks in the native range of *R. ponticum* will be more connected and interactions will be distributed more evenly when compared to networks in the plant’s introduced range.

**Hypothesis 2:** *Rhododendron ponticum* will act as a “super generalist” in insect-flower interaction networks (i.e. it will occupy a central role in the community and have the greatest interaction strength of all plant species) in its introduced range, but not in its native range.

**Hypothesis 3:** Following the logic from the enemy release hypothesis, floral larcenists will interact more strongly with *R. ponticum* in its native range than in its introduced range.

### 5.3 Materials and Methods

#### 5.3.1 Model species: *Rhododendron ponticum*

*Rhododendron ponticum* L. (Ericaceae), a perennial evergreen shrub, was artificially introduced to Britain and Ireland in the late eighteenth century as an ornamental species, and subsequently became severely invasive (Cross 1975, Rotherham 2001). It invades Irish heaths, bogs and woodlands, growing in dense stands or as large single individuals that can alter native plant community composition, light availability and prevent regeneration of native species (Cross 1981, Dehnen-Schmutz et al. 2004). *R. ponticum* is native to the Iberian peninsula and the Black Sea coast, where in contrast it is considered endangered; it is a ‘Red Data Book’ species in Bulgaria and is listed as at risk of extinction and legally protected in Spain (Colak et al. 1998, Ojeda et al. 2000). Invasive *R. ponticum* in the British Isles largely originates from Iberian populations (Milne and Abbott 2000). *R. ponticum* presents inflorescences of pink-purple zygomorphic flowers that produce copious sugar-rich nectar. It is visited by a variety of generalist insect visitors in its introduced and native range and requires pollination to facilitate outcrossing and improve seed set (Stout et al. 2006, Stout 2007b).
5.3.2 Study sites

The study was carried out at eight sites containing *R. ponticum*, four in each of its native and introduced range (Appendix E, Figure E.1, Table 5.2). Native sites were located in southern Spain in the Aljibe Mountains (within the Parque Natural Los Alcornocales), where *R. ponticum* occurs as a tertiary relict in deciduous *Quercus* woodlands. Sites in the introduced range were located in invaded oak or mixed woodlands in Co. Wicklow, southeast Ireland (Figure E.1). All sites were defined as 100 x 50 m areas incorporating the portion of the forest invaded by *R. ponticum* as well as any edge habitat that bordered the invaded area (see Chapter 4 for details on site boundaries). *R. ponticum* covered approximately one third of the area of native sites (determined using 20 m X 20 m quadrats, average cover 31.5% ± 9.6 SD), and sites in the introduced range were specifically chosen that contained similar coverage (average 33.2% ± 8.2 SD, Appendix E, Figure E.2). Sites within each range were similar in aspect, elevation and were at least 2 km apart, in an effort to reduce the possible overlap of pollinator communities (Knight et al. 2005a, Table 5.2).
Table 5.2 Populations used in this study: position, elevation (m above sea level), surrounding habitat type and the dates during which focal observations of the plant community were carried out.

<table>
<thead>
<tr>
<th>Location</th>
<th>Population</th>
<th>Position (m)</th>
<th>Elevation (m)</th>
<th>Habitat type</th>
<th>Dominant non-Rhodo plant species</th>
<th>Dates sampled (Year 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Alcornocales, Spain</td>
<td>Garganta de Puerto Oscuro (N1)</td>
<td>36.518 N -5.632 W</td>
<td>605</td>
<td>Stream valley, <em>Quercus canariensis</em> dominant canopy</td>
<td><em>Orchis langei, Teucrium fruticans, Lavandula stoechas subsp. stoechas</em></td>
<td>May 4, 10, 13</td>
</tr>
<tr>
<td></td>
<td>Garganta del Aljibe (N2)</td>
<td>36.538 N -5.635 W</td>
<td>469</td>
<td>Stream valley, <em>Q. canariensis</em> and <em>Quercus suber</em> dominant canopy</td>
<td><em>Digitalis purpurea ssp. bocquetii, Galactites tomentosa, L. stoechas</em></td>
<td>May 3, 8, 12</td>
</tr>
<tr>
<td>El Palancar Spring (S1)</td>
<td></td>
<td>36.081 N -5.543 W</td>
<td>495</td>
<td>Stream valley, steep SW facing slope. On edge of <em>Q. suber</em> forest</td>
<td><em>Ranunculus bulbosus ssp. adscendens, Matricaria chamomilla, D. purpurea</em></td>
<td>May 5, 7, 11, 14</td>
</tr>
<tr>
<td>Llanos del Juncal (S2)</td>
<td></td>
<td>36.105 N -5.540 W</td>
<td>747</td>
<td>Cloud forest, <em>Q. canariensis</em> and <em>Crataegus monogyna</em> dominant canopy</td>
<td><em>Allium triquetrum, Ulex borgiae, O. langei</em></td>
<td>May 6, 9, 14</td>
</tr>
<tr>
<td>County Wicklow, Ireland</td>
<td>Crossover</td>
<td>52.894 N -6.400 W</td>
<td>165</td>
<td>Riparian woodland, streamside, <em>Quercus petraea</em> and <em>Betula pendula</em> dominant canopy</td>
<td><em>Hyacinthoides non-scripta, Vicia sepium, Ulex europaeus</em></td>
<td>May 27, 31, June 2</td>
</tr>
<tr>
<td></td>
<td>Dunran</td>
<td>53.060 N -6.102 W</td>
<td>160</td>
<td>Mixed forest plantation, valley, <em>Pinus contorta</em> and <em>Quercus pendula</em> dominant canopy</td>
<td><em>Veronica chamaedrys, Glechoma hederacea, Digitalis purpurea</em></td>
<td>May 24, 28, June 3, 8, 13</td>
</tr>
<tr>
<td></td>
<td>Shankhill</td>
<td>53.192 N -6.427 W</td>
<td>284</td>
<td>Mixed forest plantation, streamside, <em>Fagus sylvatica, Fagus excelsior</em> and <em>P. contorta</em> dominant canopy</td>
<td><em>Stellaria holostea, V. chamaedrys, Ranunculus repens</em></td>
<td>May 23, 25, 29, June 6, 23, 28</td>
</tr>
<tr>
<td></td>
<td>Trooperstown</td>
<td>53.017 N -6.274 W</td>
<td>185</td>
<td>Mixed forest, <em>Q. pendula, Fraxinus excelsior &amp; B. pendula</em> dominant canopy</td>
<td><em>V. chamaedrys, R. repens, H. non-scripta</em></td>
<td>May 30, June 1, 7, 9</td>
</tr>
</tbody>
</table>
5.3.3 Sampling insect and plant communities

In 2011, each site was sampled during peak *R. ponticum* flowering on three distinct days in the native populations in Spain (3-14 May) and the introduced populations in Ireland (24 May – 28 June, Table 5.2). We sampled insect-flower communities using timed observations (Morales and Aizen 2006, Olesen et al. 2008, Kaiser-Bunbury et al. 2011) as opposed to the transect method (Memmott 1999, Lopezaraiza–Mikel et al. 2007, Bartomeus et al. 2010). In order to reduce the effects of diurnal variation in visitation patterns, plant species were observed for 3 x 10 min sessions each day, once in each the morning (9:00-12:00), midday (12:00-14:30), and afternoon (14:30-17:30). We aimed to observe each plant species for a total of 1.5 h/site, but inclement weather and differences in flowering phenology reduced total observation time/species to an average of 1.15 h±0.49 h (SD). Given the short flowering period of *R. ponticum*, we expect that the majority of insect-flower interactions occurred in a relatively limited time frame that was sufficiently captured through our sampling effort. Furthermore, Hegland et al. (2010) demonstrated that it is possible to capture the majority of functionally important pollinator species with limited sampling effort. Observations were carried out on dry days when the temperature was > 12°C and wind speeds were ≤ 4 according to the Beaufort Scale. We expressed pollinator activity as visitation rate (visits/flower/hour) to account for differences in observation time and number of flowers observed of different plant species (Kaiser-Bunbury et al. 2011).

During timed observations, we recorded the identity of all insect visitors that made contact with flowers of focal shrubs, treelets, or herbs. A visit (synonymous with interaction) was defined as any contact between the flower and the insect (Lopezaraiza–Mikel et al. 2007). Flowering plant species were observed independently and the number of floral units observed during each census was recorded. In this study we defined a floral unit as a single flower head, or part of a multiple head, from which a medium-sized bee has to fly rather than walk to reach another floral unit of the same species (Dicks et al. 2002). We recorded the number of floral units visited, whether the visitor was collecting nectar or pollen, and whether it came into contact with the reproductive organs.

Due to National Park regulations in Spain, it was not possible to capture and kill all flower visitors for taxonomic identification. Due to this limitation, our pollinators were classified on the wing as one of 13 taxonomic groups: ants, bumblebees, Dermaptera, Hemiptera, Lepidoptera, Orthoptera, pollen beetles, solitary bees/wasps, spiders, Syrphids, Thysanoptera, other Coleoptera and other Diptera. This limitation means that the networks we observed and
the network level and species level parameters we calculated are not comparable to those from studies with high taxonomic resolution. Nonetheless, our taxonomic approach has been utilized in similar studies (Ferrero et al. 2013) and was identical in the native and introduced range making our networks comparable. Furthermore, using taxonomic groups in a biogeographical comparison of plant-insect communities may be a favourable alternative to species-level data; this approach allows for the comparison of key groups of flower visitors even though the identity of individual species differs between regions. In Ireland, flowering plant identification followed Webbs Irish Flora (Parnell and Curtis 2012) and Rose (2006). In Spain, flowering plants were identified using the Flora de Andalucia (Aparicio et al. 1987). Reference specimens from both regions are deposited at Trinity College Dublin.

At each site, we utilized a stratified random approach reflecting the cover of *R. ponticum* (approximately one third) to quantify the relative floral abundance of each plant species. To sample floral abundance in the area covered by *R. ponticum*, 1 x 1 meter quadrats were placed approximately 1m from the ground on 12 randomly selected *R. ponticum* individuals and the number of floral units in each was counted. In addition, eight 10 m transects were established in areas free from *R. ponticum* cover and the number of floral units of each flowering species was recorded in three 1 x 1 m quadrats along each transect (at 0, 5 and 10 m, total 24 quadrats). Floral abundance sampling was carried out three times at each site at the same time insect observations were made. Because of limited time and resources, rare flowering plant species (found in <5 quadrats/site) were excluded from timed observations. Abundance data were combined for each sampling period and floral abundance, the mean number of flowers per cubic meter, was calculated for each species by dividing the total number of flowers by the total number of quadrats sampled at each site.

### 5.3.4 Insect-flower interaction networks

To visualize insect-flower interaction webs, bipartite interaction networks were plotted using visitation data from each site (Dormann et al. 2008). In constructing our matrices, we used visitation frequencies to represent interaction strength (sensu Kaiser-Bunbury et al. 2011), and quantified visits based on the floral abundance of the interaction partner. Thus 'mean interaction frequency' was represented as the total number of visits/flower/hour of animal species *a* to plant species *p* multiplied by the floral abundance (avg. floral units/m²) of plant species *p* (Vázquez et al. 2005, Bascompte et al. 2006, Vázquez et al. 2007, Kaiser-Bunbury et al. 2011). Due to the small size of networks, data from each of the three visits to a site were combined and networks and network parameters were calculated at the site level (Stout and
Casey 2014). In order to compare the structure of the insect-flower interaction networks between the native and introduced ranges of *R. ponticum*, we calculated five quantitative network descriptors using the "network level" command (Dormann et al. 2008) in the bipartite package for R (version 3.0.2, R-Development-Core-Team, 2007): quantitative connectance, interaction evenness, generality, vulnerability and $H'_2$ (see Chapter 4 for calculations and definitions of network level parameters). In addition, we used the "species level" command (Dormann et al. 2008) to calculate four additional parameters for *R. ponticum* in each network: partner diversity (Shannon diversity of the interactions of each species), number of effective partners, species degree (sum of interactions per species), and strength (the sum of dependencies of each species) (Bascompte et al. 2006).

5.3.5 Flower-visiting larcenists and mutualists of *R. ponticum*

While previous studies have investigated the pollination ecology of *R. ponticum* in its native and introduced range (Stout 2007b, Stout 2007a), floral larcenists of this species have been largely ignored. In this study, we 1.) carried out single visit experiments to distinguish which taxa performed legitimate, i.e. pollen depositing, visits to *R. ponticum*, 2.) quantified visitation by floral larcenists vs. mutualists to *R. ponticum* in the native and introduced range and 3.) compared the frequency of nectar robbing on *R. ponticum* plants in both ranges.

Single visit experiments provide quantitative information about conspecific pollen deposition from flower visitors and are a good estimate of pollinator effectiveness (Stout 2007a, King et al. 2013). We performed single visit experiments at one site in the introduced range (Dunran) using the following protocol. One hundred and eleven *R. ponticum* flowers were emasculated prior to anthesis (to avoid contamination of stigmas with self pollen) and double bagged with bridal veil material to exclude insect visitors. Once emasculated flowers were fully open and stigmas were receptive, the bridal veil was removed and the flower was observed until a single insect visited. After the visit was complete, the stigma from that flower was removed, mounted on a microscope slide and stained with 0.5% safranin dye, and the number of *R. ponticum* pollen grains counted. Hoverflies of the genera *Syrphus*, *Parasyrphus*, *Melanostoma* and *Platycheirus* and pollen beetles (*Meligethes aeneus*) visited emasculated flowers naturally. For ants, an emasculated flower was held near an inflorescence of *R. ponticum* flowers upon which ants were already feeding until an individual visited, fed on nectar, and exited the flower. For bumblebees, individuals foraging on *R. ponticum* were captured in a small plastic pot and allowed to acclimate for five minutes. An emasculated flower was then presented to the bee, which was allowed to forage for nectar or pollen, and the stigma was removed after it finished
and flew off. Single visit experiments were not carried out for the remaining functional groups because of the rarity of visits from these insects to *R. ponticum*. This procedure was repeated until at least *n*=15 visits were procured from each functional groups (except pollen beetles, *n*= 5, because visitation was rare in the introduced range). Stigmas to which *R. ponticum* pollen was applied directly using a paintbrush were collected as positive controls, and stigmas that received no visits were collected as negative controls.

We categorized the 10 functional groups that were observed visiting *R. ponticum* during flower observations as either mutualist or antagonist flower visitors. This assessment was based on 1.) the frequency with which insects contacted the anthers or stigma or collected nectar or pollen from *R. ponticum* during focal observations, 2.) potential for morphological mismatching with *R. ponticum* flowers (i.e. does the size and behaviour of insects indicate that they will effectively pollinate *R. ponticum* flowers) and 3.) data from single visit experiments (Table 5.3). All timed observations of *R. ponticum* at a site were combined, and the total number of antagonist visitors was divided by the total number of mutualist visitors to calculate a ratio of antagonist: mutualist visits for each site. In addition, Shannon evenness of functional groups visiting *R. ponticum* was calculated at the site level. Finally, every patch of *R. ponticum* that was surveyed for insect visitors was inspected at the end of the observation period for evidence of nectar robbing (nine patches of *R. ponticum* flowers per site, *n*= 36 patches in each the native and introduced range). We noted the number of flowers that had holes in the base of the corolla near the nectary (Inouye 1980, Maloof and Inouye 2000, Richardson 2004); in total 1350 and 1196 flowers were inspected in surveys for nectar robbing in the native and introduced range respectively, (average 33 flowers ±13 per survey).
<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>% visits contacting stigma/anthers(^1)</th>
<th>% visits collecting nectar/pollen(^1)</th>
<th>Morphologically mismatched?(^2)</th>
<th>Average # pollen grains deposited on stigma(^3)</th>
<th>Final classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ants</td>
<td>5.41</td>
<td>40.54</td>
<td>Yes</td>
<td>0.067</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Bumblebees</td>
<td>95.93</td>
<td>95.93</td>
<td>No</td>
<td>26.46</td>
<td>Mutualist</td>
</tr>
<tr>
<td>Hemiptera</td>
<td>0.00</td>
<td>0.00</td>
<td>Yes</td>
<td>NA</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>100.00</td>
<td>100.00</td>
<td>Sometimes</td>
<td>NA</td>
<td>Mutualist</td>
</tr>
<tr>
<td>Pollen beetles</td>
<td>73.30</td>
<td>75.57</td>
<td>Yes</td>
<td>0</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Solitary bees</td>
<td>39.02</td>
<td>34.15</td>
<td>No</td>
<td>NA</td>
<td>Mutualist</td>
</tr>
<tr>
<td>Syrphids(^4)</td>
<td>51.75</td>
<td>18.18</td>
<td>Sometimes</td>
<td>0.5</td>
<td>Mutualist</td>
</tr>
<tr>
<td>Thysanoptera</td>
<td>0.00</td>
<td>50.00</td>
<td>Yes</td>
<td>NA</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Other coleoptera</td>
<td>47.06</td>
<td>41.18</td>
<td>Sometimes</td>
<td>NA</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Other diptera</td>
<td>34.82</td>
<td>33.93</td>
<td>Yes</td>
<td>NA</td>
<td>Antagonist</td>
</tr>
</tbody>
</table>

\(^1\) Observations made during observations of *R. ponticum*. \(^2\) Determined by size and behaviour of flower visitors. \(^3\) Results from single visit experiments. \(^4\) Pollen deposition based on visits from a combination of *Syrphus, Parasyrphus, Melanostoma* and *Platycheirus* only.

### 5.3.6 Data analysis

Quantitative network-level and species-level parameters were compared between the two ranges using Welch’s unpaired \(t\) tests. Likewise, differences in the ratio of antagonist:mutualist visitors, Shannon Evenness of functional groups and the abundance of insects from each functional group visiting *R. ponticum* were compared between ranges using an unpaired \(t\) test. The limited power associated with out low sample size (\(n = 4\) networks per range) is justified by the effort involved in sampling entire insect-flower interaction communities in the limited time period during which *R. ponticum* is in flower, and is similar to other studies of invasive plants (Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2010, Ferrero et al. 2013).

We also compared flower visitor diversity and abundance in the two ranges by using only visitation data from *R. ponticum* and the three most abundant co-flowering plant species at each site (Table 5.2). Sampling effort (total observation time, as well as time spent observing *R. ponticum* vs. heterospecific species at each site) was therefore standard, allowing for a direct comparison of the number of insects observed. We calculated site-level insect taxonomic group richness, overall abundance of flower visitors, abundance of visitors to *R. ponticum* and abundance of visitors to the native species. Data were analyzed for differences between the two ranges using Welch’s unpaired \(t\)-tests. All analyses were carried out in R, version 3.0.2 (R Development Core Team 2011).
5.4 Results

5.4.1 H1- Insect-flower interaction network structure in the native vs. introduced range

In nearly 95 h of observations, a total of 2,117 insect-flower interactions were observed, 994 in _R. ponticum_’s native range and 1,123 in its introduced range. The identity of the most dominant insect functional groups varied between the two ranges. In Spanish sites, 32% and 19% of all individual flower visitors observed were pollen beetles (_M. aeneus_) and ants respectively, while in Irish sites 41% of visitors were Syrphids, 26% were other Diptera and 16% were bumblebees (Figure 5.1). In both ranges however, even though bumblebees were not the most abundant function group, they visited many individual flowers during each observation period, and thus accounted for the largest percent of overall visits (Appendix E, Figure E.3).

During focal observations, 20 and 25 unique flowering plant species were surveyed in the native and introduced ranges respectively. Even though the identity of the co-flowering plant species differed between the two ranges, the plant communities were similar in structure; in both locations, _R. ponticum_ grew in dense stands in moist soil and covered approximately one third of the site (Figure E.2), while co-flowering species grew at the edges of sites that were free from _R. ponticum_ cover. One species, _Digitalis purpurea_, was commonly found co-occurring with _R. ponticum_ in both ranges, present at 3/4 of each the native and introduced sites. _R. ponticum_ flowers were always the most abundant floral resource in the introduced populations; the floral abundance value of the invasive species was on average 7.11 times (± 4.03 SD) greater than the next most abundant, non-Rhodo flowering species (Table 5.2). In the native populations, _R. ponticum_ flowers were the most abundant in 3/4 sites, and values were on average 3.60 times greater (± 2.37 SD) than the next most abundant flowering plant species. Site level _R. ponticum_ floral abundance was significantly higher in the introduced vs. the native range (independent t-test, t =3.61, d.f. =4, P = 0.0226), but total floral abundance did not vary significantly (t = -0.9954, d.f. = 5.992, P = 0.358).

Overall, insect-flower interaction network structure was similar for communities in _R. ponticum_’s native and introduced range (Figure 5.1). Networks from both locations were relatively small (8-10 plant and 9-11 insect functional groups in Spanish populations, 10-13 plant and 10-11 insect functional groups in Irish populations), but network size (a qualitative parameter, calculated as plant spp. x animal spp.) in introduced networks was larger (Table 5.4). The quantitative network parameters calculated for sites in both ranges were also
similar: quantitative connectance, interaction evenness, vulnerability and $H^2$ did not vary significantly between the native and introduced range, but generality, the weighted mean number of plant species per visitor species, was lower in the native range than the introduced range (Table 5.4).

Table 5.4 Comparison of the mean quantitative network level and (for *R. ponticum*) species level parameters in the native (Spanish) and the introduced (Irish) range, averaged across sites. Significant differences indicated by ** (Welch's unpaired t test, $P < 0.05$).

<table>
<thead>
<tr>
<th>Network Parameter</th>
<th>Spanish Average</th>
<th>Irish Average</th>
<th>Test statistic (t)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative connectance</td>
<td>0.19</td>
<td>0.18</td>
<td>1.33</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Interaction evenness</td>
<td>0.62</td>
<td>0.59</td>
<td>1.01</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Generality</td>
<td>3.09</td>
<td>4.21</td>
<td>3.30</td>
<td>&lt;0.01**</td>
</tr>
<tr>
<td>Vulnerability</td>
<td>3.90</td>
<td>3.34</td>
<td>-1.34</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>$H^2$</td>
<td>0.32</td>
<td>0.27</td>
<td>-0.89</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Partner diversity</td>
<td>1.47</td>
<td>1.32</td>
<td>-1.13</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Number of effective partners</td>
<td>4.43</td>
<td>3.79</td>
<td>-1.15</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Species degree</td>
<td>7.25</td>
<td>6.25</td>
<td>-1.85</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Species strength</td>
<td>4.45</td>
<td>3.61</td>
<td>-2.08</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Network size</td>
<td>82.50</td>
<td>115.00</td>
<td>5.02</td>
<td>&lt;0.01**</td>
</tr>
</tbody>
</table>
Figure 5.1, a-d  Insect-flower interaction networks for communities containing *R. ponticum* in its native range (southern Spain, a.-d.).
Figure 5.1, e-h Insect-flower interaction networks for communities containing *R. ponticum* in its introduced range (Ireland, e.-h.). Throughout the figure, for each web, upper bar widths represent pollinator guild abundance while lower bar widths are determined by the interaction strength with insect species. Linkage width indicates the frequency of the interaction. One network was created for each site.
5.4.2 H2- *R. ponticum'*s role in native vs. introduced insect-flower communities

*R. ponticum* dominated insect-flower interaction networks in both its native and introduced range (Figure 5.1). The species level parameters calculated for *R. ponticum* indicate that it is playing a similar role in network structure in both ranges; partner diversity, number of effective partners, species degree and species strength were not significantly different in Spanish vs. Irish networks (Table 5.4). *R. ponticum* was the plant with the highest value for species degree (the sum of interactions per species) in 3/4 native networks and 2/4 introduced networks, indicating its dominant role in interaction network structure. Furthermore, in all eight networks, it was the plant species with the highest species strength (the sum of dependencies of each species, Bascompte et al. 2006).

When only observations of *R. ponticum* and the three most abundant co-flowering species were considered, neither the richness of insect functional groups (Spanish average: 9.5 ±1.3, Irish average: 9.0 ±1.4) nor the abundance of insect species (Spanish average: 189 ± 40, Irish average: 172 ± 29) differed significantly between ranges (richness: t = -0.52, d.f. = 5.95, *P* = 0.62; abundance: t = -0.70, d.f. = 5.41, *P* = 0.51). The average percentage of the total interactions that included *R. ponticum* were similar in both ranges; 47% (± 14.6) and 53% (± 13.3) in Spanish and Irish populations respectively. Similarly, the number of visits to *R. ponticum* and to the three co-flowering plant species did not differ significantly between ranges (*R. ponticum*: t = 0.40, d.f. = 4.61, *P* = 0.70; co-flowering spp.: t = -0.88, d.f. = 4.74, *P* = 0.42, Figure 5.3).
Figure 5.3 Mean flower-visitor diversity and abundance in introduced (Irish) and native (Spanish) communities comprised of *R. ponticum* and the three most abundant co-flowering plant species. 

- **a.** Guild richness of flower visitors, 
- **b.** Overall insect visitor abundance, 
- **c.** Insect visitor abundance to *R. ponticum*, 
- **d.** Insect visitor abundance to co-flowering species. 

Values averaged across sites.
5.4.3 H3- Flower-visiting larcenists and mutualists of *R. ponticum*

The ratio of antagonist: mutualist flower-visitors to *R. ponticum* was significantly higher in the native range when compared to the introduced range (\(t = -3.78, \text{ d.f.} = 3.419, P = 0.0258\), Figure 5.4). This pattern can be attributed mostly to a difference in visitation from specific taxonomic groups between the two ranges; in the introduced populations, pollinating bumblebees visited *R. ponticum* significantly more (\(t = 2.72, \text{ d.f.} = 5.69, P = 0.037\)), while pollen-thieving pollen beetles visited significantly less (\(t = -4.715, \text{ d.f.} = 5.25, P = 0.005\)). Furthermore, the number of Syrphids visiting *R. ponticum* was greater in the introduced range while the number of nectar-thieving ants was lower, although these differences were not significant (Syrphids: \(t = 1.84, \text{ d.f.} = 3.02\); Ants: \(t = -1.59, \text{ d.f.} = 4.22, P > 0.05\)). There was no difference in Shannon’s Evenness of taxonomic groups between the native and introduced range (\(t = -1.82, \text{ d.f.} = 5.99, P > 0.05\), Figure 5.4).

Evidence of nectar robbing was found in all four native populations, but the prevalence varied greatly from site to site, ranging from 0.29% at Llanos del Juncal, to 24.2% at El Palancar Spring. In contrast, evidence of nectar robbing in the introduced populations was extremely rare; only one site (Trooperstown) had *R. ponticum* flowers with holes at the base of the corolla, and even there the percent of robbed flowers was low (0.80%). The insect guilds responsible for the robbing were not identified in either range, however in native populations ants often acted as secondary robbers.

![Figure 5.4](image-url) Comparison of mean ratio of antagonist:mutualist flower- visitors (dark grey) and Shannon Evenness of flower-visiting insect functional groups (light grey) to *R. ponticum* in the native (Spanish) and introduced (Irish) ranges, averaged across sites. Significance at \(\alpha = 0.05\) level. Bars represent standard errors, \(n = 4\) populations per range. Shannon Evenness was calculated using insect abundance, i.e. number of visitors observed during flower surveys of *R. ponticum*. 101
5.5 Discussion

5.5.1 H1 &2: Insect-flower interaction networks in the native vs. introduced range

This study is the first to use a biogeographical approach to compare the structure of insect-flower interaction networks in the native and introduced range of an invasive plant. Although the species composition of communities was predictably different in the two locations, the quantitative parameters that describe the structure of the interaction networks remained similar. Only generality, the weighted linkage for insect visitors, increased significantly in the introduced range. Although increased generality is often associated with an increase in the diet breadth of flower visitors (Memmott et al. 2004), in our case it was instead likely due to a slight increase in network size in the introduced range (Jordano 1987).

Previous studies have found that the presence of an invasive plant can significantly alter native plant-pollinator network structure. For example, network nestedness increased in plots with low levels of invasive Opuntia stricta when compared to paired uninvaded plots in a native Mediterranean plant community (Bartomeus et al. 2008). This study concluded that differences in network structure occurred because the invasive plant did not coevolve with the members of the native community (Bartomeus et al. 2008). If this were the case, we would expect network structure to differ fundamentally in communities in which plants are native vs. those where they are introduced. On the contrary, our results demonstrate that in the case of R. ponticum, overall network structure is similar, regardless of the origin (native vs. invasive) of the plant. Our results are also in congruence with previous studies that examined R. ponticum in invaded and uninvaded sites in its introduced range. This work found that although the alien R. ponticum plays a central role in networks, insect-flower interaction network structure is robust to the introduction of invasive plants (Dietzsch 2009, Vilà et al. 2009). Still, invaded vs. uninvaded comparisons do not address the issue of the invasive plant’s origin; a biogeographical approach such as ours is thus needed to compliment studies such as these.

Although many invasive plant species have been shown to become well integrated and dominant in plant-pollinator networks in their introduced ranges (Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2008, Bartomeus et al. 2010), our study is the first to test whether this pattern is consistent when the invasive species has coevolved with the other members of its native community. Contrary to our expectations, R. ponticum played a dominant role in insect-flower interaction network structure in both its native and introduced range. It had the highest species strength of any plant species in all eight networks and was strongly connected
to most insect functional groups. In addition, when only *R. ponticum* and the three most abundant co-flowering species were observed, it received approximately half of all insect visits regardless of the range. Taken together, these results indicate that *R. ponticum* acts as a "super generalist" plant species (Bartomeus et al. 2008) in both native and introduced insect-flower interaction networks.

Many invasive plants, including *R. ponticum*, have large, generalist flower displays with easily accessible nectar or pollen rewards that attract a variety of visitors (Memmott and Waser 2002, Stout et al. 2006). Plants with these floral characteristics may often dominate network structure regardless of their range, especially if they are highly abundant (Carvalheiro et al. 2014). In fact, it is possible that these particular floral characteristics contribute to the success of certain invasive plant species, enabling them to establish mutualisms with many generalist pollinators in introduced ranges (Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2010). Our results suggest that invasive plants such as *R. ponticum* are not dominant necessarily because they are novel introductions, but because of the attractive and generalist nature of their floral displays.

5.5.2 Flower-visiting larcenists and mutualists of *R. ponticum*

We found higher visitation from floral larcenists and an increased incidence of nectar robbing in *R. ponticum*’s native range when compared to its introduced range, however we were not able to identify primary nectar robbers during focal observations. *Bombus terrestris* and *Xylocopa violacea* have previously been implicated in nectar robbing in the region (Guitian et al. 1994) and were present in Spanish *R. ponticum* populations, but robbing behaviour was never observed. Likewise, *Bombus* species may rob *R. ponticum* nectar in Irish populations (Irwin and Brody 1999), although we only ever observed legitimate visits from members of this genus and bumblebees are known to be the main pollinators of *R. ponticum* in its introduced range (Stout 2007a). Ants acted as secondary nectar robbers in both ranges, feeding from the holes left from primary robbers (Inouye 1980).

Floral larcenists can have negative impacts for plant fitness (Inouye 1980, Irwin and Brody 1999, 2000, but see Stout et al. 2000, Richardson 2004). Similar to folivores and plant diseases, it is possible that these plant "enemies" could contribute to population control in the native range, and that enemy release could aid in the success of the invasive plant in the introduced range (Keane and Crawley 2002, Colautti et al. 2004). Although our finding of decreased floral larceny in introduced populations of *R. ponticum* is intriguing in this regard,
increased prevalence or damage from enemies does not necessarily translate to enemy release. Further studies comparing the impacts of floral larcenists for *R. ponticum* fitness in both ranges are required (Siemann and Rogers 2003, Colautti et al. 2004, Hierro et al. 2005). Our study, however, is the first to our knowledge to investigate any type of floral larceny in the context of enemy release.

The decreased prevalence of floral larcenists visiting *R. ponticum* in introduced populations is especially intriguing in light of the chemical make-up of the nectar of this invasive plant. *R. ponticum* contains grayanotoxins, diterpenes known for their toxicity to mammals (Koca and Koca 2007) in its floral nectar (Tiedeken et al. 2014). The "nectar robber hypothesis" was one of the first explanations proposed to rationalize the paradoxical phenomenon of "toxic nectar;" plants may produce toxins in their nectar that deter antagonist flower visitors, such as nectar robbers or thieves, but not legitimate pollinators (Janzen 1977, Baker et al. 1978, reviewed by Adler 2000). Floral larcenists in the introduced range may be less tolerant to nectar grayanotoxins than those in the native range, possibly explaining the decreased visitation we observed and providing a potential mechanism for enemy release from floral larcenists. However in the introduced range, the toxins in *R. ponticum* nectar have been found to deter and poison native honeybees and some solitary bees that could provide pollination services (Chapter 3). Concentrations of nectar toxins in this invasive species may thus be under conflicting selection pressures in its introduced range (Adler and Irwin 2005); a biogeographical comparison of toxin concentrations would aid in understanding these evolutionary pressures, and more research in this area is clearly needed (Egan 2014).

5.5.3 Challenges of using a biogeographical approach for community level studies

Community level biogeographical studies comparing insect-flower interaction networks in the native and introduced range of an invasive plant present a unique set of challenges. Sampling entire plant-pollinator communities from geographically disparate locations is time-intensive and requires extensive taxonomic expertise. Because of the nature of these studies, sites or populations will inevitably be nested within range (Blaisdell and Roy 2014), and as a result, as many native and invasive populations should be sampled as possible in order to increase statistical power. Sampling plant-pollinator communities using a transect approach may therefore be preferable to timed observations, because transects are ideal when time is limited and when replicate sites need to be sampled (Gibson et al. 2011). In forest habitats, *R. ponticum* grows in dense stands or clumps (Cross 1981), making habitats heterogeneous in nature and therefore difficult to survey using transects. Our use of timed observations,
however, limited the number of sites we could realistically cover in the short period of peak *R. ponticum* flowering, reducing statistical power. Future biogeographical studies should focus on herbaceous invasive species that grow in homogenous habitats with co-flowering plants, so that the transect method can be utilized.

5.5.4 Conclusions

The field of invasion ecology is fraught with assumptions; for example, it is often assumed that invasive species are more abundant and perform better in regions where they are exotic than in their native ranges (Hierro et al. 2005). As more detailed demographic studies are carried out however, these assumptions are being challenged; some studies indicate that invasive plants are equally successful or possess similar traits in both native and exotic ranges (Buckley et al. 2003, Paynter et al. 2003). Our study likewise suggests that when it comes to the role invasive plants play in plant-pollinator interaction networks, the situation may be similar in introduced and native populations. We demonstrated that *R. ponticum* dominates insect-flower interaction networks regardless of its range; in other words, it dominates communities in the introduced range not because it is exotic, but because that is its nature, even in communities in which it is native. It is clear that studying invasive species in a community context in their native as well as their introduced ranges can provide useful insights to the fields of both invasion and community ecology.
Chapter 6

General Discussion
6 General Discussion

In this thesis I aimed to better understand how a poorly studied potential driver of pollinator decline, invasion by introduced plant species, impacts native pollinators. Furthermore, I considered how a specific plant trait, the presence of nectar secondary compounds, modulates the impacts of plant invasion for native pollinators. I used invasive Rhododendron ponticum as a model system for my studies and carried out extensive laboratory assays and field surveys on a variety of scales to investigate how pollinators respond to invasion by an introduced plant containing secondary compounds in its nectar. In Chapter 2, I found that a generalist bumblebee has poor acuity for detection of nectar secondary compounds, which may have implications for both pollinators and plants. Chapter 3 demonstrated that the nectar secondary compounds from invasive R. ponticum have species-specific effects for ecologically and economically valuable native pollinators, which can modulate pollinator responses to plant invasion. I used a community-level approach in Chapter 4 to determine that although R. ponticum is well-integrated into insect-flower interaction networks in its introduced range, communities are able to cope with dramatic seasonal changes in floral resources. Finally, in Chapter 5 I carried out a biogeographical comparison of insect-flower interaction networks in R. ponticum's native and introduced range and found that network structure was similar in both locations. In this final chapter, I will synthesize and put my findings into context given the relevant literature in the field, discuss methodological considerations and finally suggest some areas that require future research.

6.1 Implications of plant invasion for pollinators

Invasion by alien plants often leads to a reduced diversity of native plant species via indirect or direct mechanisms, and can ultimately reduce overall biodiversity (Martin 1999, Sala et al. 2000). Extensive monocultures of introduced plants commonly replace native vegetation (Ernst and Cappuccino 2005). As a result, many studies on plant invasion focus only on the impacts on native plant communities (Levine et al. 2003, Traveset and Richardson 2006). In contrast, until recently studies of the impacts of plant invasion on arthropods were practically nonexistent (e.g. Toft et al. 2001, Ernst and Cappuccino 2005, Fork 2010, Spafford et al. 2013), and investigations specifically examining pollinating insects are still rare (but see Appendix B). This is particularly surprising given that plant invasion is likely to impact a variety of resources that are essential to pollinators (Bjerknes et al. 2007, Stout and Morales 2009). Although my thesis mainly focused on changes in forage resource availability (Chapter 2, 3 and 4), plant
invasion can impact native pollinators directly or indirectly via changes to any essential resources, e.g. nesting, mating and overwintering sites (Edwards and Jenner 2005, de Groot et al. 2007, McKinney and Goodell 2010). This fact is recognized in the literature on pollinator decline, because invasive alien species are commonly cited as a primary contributor to pollinator losses (Potts et al. 2010b, Gonzalez-Varo et al. 2013, Vanbergen and Insect Pollinators Initiative 2013). Yet basic questions remain unanswered, including clarity on the direction of the impacts of plant invasion for native pollinators. My thesis contributed to these knowledge gaps. Collectively, my work highlights the fact that the directionality of the impact of invasive plants for pollinators may vary between taxa, and can be modulated by plant traits (Chapter 3) such as reward quality (Chapter 2 and 3), and the composition of the insect-flower interaction network at invaded sites (Chapter 4 and 5).

6.1.1 Direction of impacts of plant invasion for pollinators

It is often assumed that plant invasion negatively impacts pollinators (Litt et al. 2014), however in reality the direction of impacts can vary, depending on how resources essential to pollinators are affected (Rodriguez 2006, Stout and Morales 2009, Chapters 1 and 3). A recent meta-analysis by Montero-Castaño and Vila (2012) attempted to better understand the directionality of the impacts of biological invasions for native pollinators. One of the main goals was to determine whether pollinators exhibited taxonomic differences in their response to invasion; in other words, do some pollinator groups exhibit greater resistance or susceptibility to biotic invasions than others (Montero-Castaño and Vila 2012)? Their study found that both pollinator visitation rates and species richness differed in their response to invasion depending on the taxa being examined, but the taxonomic groups for which there were sufficient studies to answer this question were limited to “bees” and “other insects.” My work demonstrates that even within one of Montero-Castaño and Vila’s (2012) taxonomic groups (bees), there can be variation in the direction of impacts of plant invasion for pollinators (Chapter 3).

My work demonstrated that 1.) GTXs do not render *R. ponticum* nectar unpalatable for the bumblebee *B. terrestris* (Chapter 2), 2.) nectar GTXs do not negatively impact the survival of *B. terrestris*, even in combination with additional stressors (Chapter 3), and 3.) in the wild, *B. terrestris* (as well as other bumblebee species) are common visitors of *R. ponticum* (Chapters 4 and 5). The nectar and pollen provided by invasive *R. ponticum* is therefore an important forage resource for native Irish generalist bumblebee species, especially in areas lacking in floral resources. This conclusion is in congruence with previous research on this system. Two
closely related bumblebees, *Bombus lucorum* and *B. pascuorum*, have higher colony densities in *R. ponticum* invaded sites when compared to uninvaded control sites (Dietzsch 2009), and bumblebees were found to be common visitors to *R. ponticum* in previous studies carried out in the invasive range (Stout et al. 2006, Stout 2007a). Studies of other systems have also found similar results; pollinators able to utilize the forage resources provided by invasive or mass-flowering crop species can experience short-term (Westphal et al. 2009) and longer-term benefits from large monocultures of these plants (Shapiro 2002, Novotny et al. 2003, Westphal et al. 2003, Tepedino et al. 2008). My work, however, demonstrates that pollinator taxa may have different responses to the resources provided by invasive plant species. In particular my studies are the first to highlight the negative responses of *A. mellifera* and *A. carantonica* to *R. ponticum* nectar (Chapter 3).

It is likely that invasive plants will provide more suitable forage resources for pollinators if their chemistry and floral traits are similar to the native plants they replace (Ernst and Cappuccino 2005). This is not always the case, because in some situations novel floral traits of invasive plants make them more attractive to native insects, e.g. higher sugar content in nectar or more nectar volume produced per unit time (Stout and Morales 2009). In the case of *R. ponticum*, however, nectar chemistry clearly presents an unacceptable barrier for some native pollinators (Chapter 3). A similar phenomenon has been found to occur in California; adult Lepidopteran species lay their eggs on invasive plants whose nectar secondary compounds are toxic to larvae (Graves and Shapiro 2003). Chapter 3, however, is the first study to my knowledge to demonstrate that an invasive flowering plant provides toxic nectar to economically and ecologically important bee species.

The existence of species or taxon specific responses to invasion by introduced plants should be taken into account (Chapter 3, Montero-Castaño and Vila 2012) because pollinators are a diverse group of organisms that require a variety of forage and nesting resources and abiotic conditions (see Chapter 1). Plant invasion may therefore increase resource availability for some pollinators but simultaneously decrease it for others, modulating the directionality of the impacts of this phenomenon. This is precisely what we found in the case of *Rhododendron ponticum* (Chapter 3); other studies have likewise found that plant invasion can have mixed impacts for different pollinator taxa (Goodell 2003, Graves and Shapiro 2003, de Groot et al. 2007, Baskett et al. 2011, Montero-Castaño and Vila 2012). For example, *Solidago canadensis*, invasive goldenrod, had a positive impact on hoverfly abundance and diversity in Slovenia, but negatively impacted butterfly abundance, species richness and diversity (de Groot et al. 2007).
Multiple studies or studies that consider spatial and temporal variation (e.g. Chapter 4 and 5) can also reveal a more complex story; invasive *Solidago* species negatively impacted pollinator populations (including hoverflies) in Poland (Moron et al. 2009), and in Slovenia hoverflies were only positively impacted during the flowering of the invasive, but were negatively impacted before flowering began (de Groot et al. 2007). The variation in the responses of pollinators to plant invasion poses considerable challenges for the prediction of the impacts of biological invasions, but it is inevitable given the diversity of traits of both pollinators and invasive plants.

6.1.2 Modulators of the response of pollinators to plant invasion

When examining the factors that contribute to the direction of pollinator responses to plant invasion, the traits of pollinators, plants and of the community should be considered (Stout and Morales 2009). First, it is often hypothesized that specialist pollinators will be negatively impacted by plant invasion because they are less likely to utilize resources provided by the invasive species (Tepedino et al. 2008, Stout and Morales 2009). In contrast, generalist pollinators are the most frequent visitors to invasive plant species (Richardson et al. 2000a, Memmott and Waser 2002, Tepedino et al. 2008), and may be more likely to benefit from the presence of abundant flowering invasive plants (Graves and Shapiro 2003, Tepedino et al. 2008, Dietzsch 2009). In parallel with these predictions, generalist *B. terrestris* utilizes and benefits from *R. ponticum* invasion (Chapter 2, 3 and 4, Stout et al. 2006, Stout 2007a, Dietzsch 2009). In addition, I observed mostly generalist Hymenoptera and Diptera species foraging on invasive *R. ponticum* in the plant's introduced range (Chapter 4 and 5). On the other hand, while generalist *A. mellifera* is often found foraging on invasive plant species (Memmott and Waser 2002, Lopezaraiza–Mikel et al. 2007, Aizen et al. 2008b, Bartomeus et al. 2010, Williams et al. 2011), the toxicity of nectar GTXs prevented associations between *R. ponticum* and *A. mellifera* in our system (Chapter 3 and 4). Specialist pollinators likewise can exhibit exceptions to this specialist-generalist paradigm, particularly when invasive plant species are similar to the native plants that the pollinators specialized on (Graves and Shapiro 2003, Tepedino et al. 2008). For example, Tepedino et al. (2008) found that *Colletes petalostemonis*, a native legume specialist, readily foraged on introduced *Melilotus* species (also legumes) in a national park in Utah.

Just as researchers search for plant traits that are functionally important in promoting invasiveness (Pyšek and Richardson 2007, Milbau and Stout 2008, Higgins and Richardson 2014), it is important to consider which traits of invasive plants will lead to particularly
negative or positive responses from pollinators (Stout and Morales 2009). These include factors such as the quality (e.g. sugar content, amino acid concentrations, secondary compounds, Chapter 2 and 3, Baker and Baker 1973, Baker and Baker 1975) and quantity of nectar and pollen rewards (Stout 2007b, Nienhuis et al. 2009), the abundance of the invasive plant species (Kaiser-Bunbury et al. 2011, Stout and Casey 2014), the attractiveness of the floral displays (Chapter 5, Bjerknes et al. 2007) and the accessibility of rewards (Corbet et al. 2001, Goulson 2003). Any one of these factors can modulate the response of pollinators to invasion by introduced plant species. The presence of toxins in floral nectar was previously not considered in this context, yet I demonstrated in Chapter 3 that this plant trait could have severe consequences for native pollinators and modulate their response to invasion. In addition, the findings from Chapter 5 demonstrate that R. ponticum’s generalist and attractive floral displays allow the plant to dominate network structure in both the native and introduced range. In contrast to toxic nectar, these plant traits are responsible for attracting generalist pollinators in the invasive range, and can therefore cause pollinators that are tolerant of the nectar secondary compounds to benefit from R. ponticum invasion (Chapter 3 and 4). This may be especially true for a perennial flowering shrub such as R. ponticum, which provides a reliable floral display each year. Annual invasive plants like I. glandulifera may have less consistent flower density or abundance and may therefore provide less consistent benefits for native pollinators (Nienhuis et al. 2009).

Finally, the responses of pollinators to plant invasion may also depend upon native community composition (Goulson et al. 2008, Stout and Morales 2009). In Chapter 4, I demonstrated that the insect-flower interaction networks at sites moderately invaded by R. ponticum do not collapse after the cessation of flowering of this invasive plant, because sufficient alternative floral resources remain to sustain most of the flower-visiting community. If the native plant community was more depauperate, as is often seen in highly invaded sites (Dietzsch et al. 2011, Stout and Casey 2014), this result could have been dramatically different (Moroń et al. 2009). Overall, I conclude that the direction of the response of pollinator taxa to plant invasion is variable due to the plethora of plant and pollinator traits and habitat conditions that can modify the establishment of mutualisms.

6.1.3 Role of biological invasions in pollinator declines

Concerns about pollinator decline are in large part driven by fear of the loss of essential pollination services (Ollerton et al. 2011, Calderone 2012). Invasive alien species are commonly listed as one of the main drivers of pollinator decline (Kearns et al. 1998, Thomson
2006, Potts et al. 2010a, Schweiger et al. 2010, Gonzalez-Varo et al. 2013), but this can include invasive animals, pathogens/parasites and predators, as well as invasive plants. The specific role that invasive plant species play in pollinator declines remains unclear, however my research demonstrates that impacts are context specific (Chapters 2, 3 and 4). For pollinators able to utilize forage resources provided by abundant invasive plants (mainly generalists), plant invasion may actually mitigate the impacts of other drivers of decline, such as the loss of flower-rich habitats (Chapter 3, 4 and 5, Schweiger et al. 2010, Gonzalez-Varo et al. 2013, Vanbergen and Insect Pollinators Initiative 2013). Alternatively, for pollinators unable to utilize novel resources (mostly specialists, although see Chapters 3 and 4), invasive plants could have non-additive, synergistic effects in regards to other drivers of pollinator decline. For these species, plant invasion is in some ways another form of habitat loss because invasive plants can change native plant community composition (Cross 1982, Pyšek and Pyšek 1995, Colak et al. 1998), and eliminate viable forage resources. However changes to insect community composition may also occur (Chapter 4) which can have knock on effects for native pollinators. Due to the asymmetric and nested nature of plant-pollinator networks (Bascompte et al. 2003, Bascompte et al. 2006), so long as invasive alien species support generalist pollinators, overall network structure is unlikely to collapse in invaded sites (Chapter 4 and 5, Memmott and Waser 2002, Memmott et al. 2004). The positive impacts of R. ponticum invasion for generalist bumblebees (Dietzsch 2009) that are able to tolerate nectar GTXs (Chapter 2 and 3) indicate that invasion by this plant species may not always result in negative pollinator responses. Indeed, recent work suggests that invasive animals, rather than invasive plants, have a more consistent negative impact on native pollinators (Montero-Castaño and Vila 2012).

In the specific case of R. ponticum, management decisions should take into consideration which taxa require conservation action. Complete removal of R. ponticum may result in detrimental impacts for B. terrestris and other GTX-tolerant bumblebees (Chapter 4), especially if they are relying on this plant during the vulnerable stages of early colony growth (Goulson 2010). Alternatively, if managers are concerned with declining honeybee (Natural Research Council 2007, Potts et al. 2010b) or potentially solitary bee populations (Potts et al. 2010a), removal of invasive R. ponticum may be advisable, given that it is replaced with native plants that provide suitable forage resources for these bees.
6.2 Implications of toxic nectar

Although studies of toxic nectar are currently popular in the field of plant-insect interactions (Adler et al. 2006, Irwin and Adler 2008, Manson et al. 2010, Adler and Irwin 2012, Adler et al. 2012, Cook et al. 2013, Manson et al. 2013), my work is among the first to consider nectar secondary compounds in an invasive plant species (but see Tadmor-Melamed et al. 2004). In this section, I will consider how GTXs specifically and nectar secondary compounds in general impact pollinators, plants and plant-pollinator communities. Throughout, I will consider what my studies can tell us about the role of nectar secondary compounds in the invasion process.

6.2.1 Impacts for pollinators

Foliar herbivores often exhibit antifeedant behaviours in response to dietary GTXs (El-Naggar et al. 1980, Klocke et al. 1991). Although the nectar of *R. ponticum* has a much lower GTX concentration than the leaves (Chapter 2, Egan 2014), I found that nectar relevant concentrations had deterrent effects for pollinating species as well (Chapter 2 and 3). Nevertheless, our community level studies demonstrated that many other generalist pollinators, especially hoverflies and bumblebees, utilize invasive *R. ponticum* nectar and pollen as a forage resource in the wild (Chapter 4, 5). This result indicates that although nectar GTXs can elicit antifeedant behaviour from some pollinator species (notably honeybees, Chapter 3), many are either not deterred by this nectar secondary compound or forage on *R. ponticum* regardless of its presence in nectar.

Interestingly, dietary GTXs elicited a range of behavioural and physiological responses from different pollinator taxa, from no detectable impacts (*B. terrestris*), to deterrence (*A. carantonica*), paralysis and/or death (*A. mellifera*, Chapter 3). Different nectar secondary compounds can collectively induce a similar variety of responses from pollinators (Pryce-Jones 1942, Detzel and Wink 1993, Cook et al. 2013, Manson et al. 2013), but it is rare that one compound is tested across several taxa using a standard protocol. It is therefore difficult to conclude whether it is common for the same nectar secondary compound to elicit such a variety of responses from different pollinator species. Extensive studies on the response of herbivores to plant secondary compounds, however, can shed some light on this issue. It is in fact common for even closely related herbivore species to have different behavioural and physiological responses to the same plant secondary compound (Berenbaum 1978, 1981, Slansky 1992). Differences in the ability of species to detoxify or sequester secondary compounds, as well as target-site insensitivity could all contribute to species-specific responses.
Overall, my findings on the impacts of nectar GTXs parallel conclusions from the literature; nectar secondary compounds can have a variety of dose and toxin dependent effects on pollinators (Manson et al. 2013).

When I began this series of studies, my original hypothesis was that nectar secondary compounds from an invasive plant would have particularly severe consequences for non-adapted pollinators in the plant's introduced range, and less serious impacts for pollinators in its native range (sensu "novel weapons hypothesis," Callaway and Ridenour 2004, see section 1.5.4). Although I was not able to explicitly test this hypothesis, my findings indirectly support it. First, solitary bees were observed visiting *R. ponticum* more commonly in its native range than in its introduced range. While this of course may have been due to other factors (e.g. different taxa, differences in abundance and species richness of solitary bee fauna), it is possible that solitary bees in *R. ponticum*'s native range are better adapted to nectar GTXs and therefore visit the plant more frequently (Chapter 3 and 5). Similarly, unlike *Apis mellifera mellifera*, honeybee subspecies in the eastern part of *R. ponticum*'s native range (*Apis mellifera caucasica* and *anatolica*) readily forage on *R. ponticum*. As a result, these bees produce GTX-containing "mad honey," which can cause life-threatening symptoms in humans if ingested (Silici et al. 2008). Although it is possible that the GTX content in *R. ponticum* nectar is simply lower in these areas, the dose response relationship established in Chapter 3 demonstrates that even half the GTX concentration found in invasive *R. ponticum* nectar causes 50% mortality in *A. mellifera mellifera*. Instead, it seems more likely that these honeybee subspecies have greater tolerance to GTXs than their western European counterparts. Finally, GTX-tolerant *B. terrestris* is native to both the introduced and native range of *R. ponticum* (Chapter 5); its coevolutionary history with *R. ponticum*, perhaps lead to its ability to cope with the plant's nectar chemistry. Further research is required to confirm this hypothesis, but my results indicate that non-adapted pollinators in the introduced range are more likely to be susceptible to toxic nectar from invasive plant species.

### 6.2.2 Impacts for plants

Even though nectar secondary compounds could have direct or indirect consequences for plant fitness (see section 1.5.2), only a few published studies consider how toxic nectar impacts plant reproduction (Adler and Irwin 2005, Kessler et al. 2008, Adler and Irwin 2012, Manson et al. 2013). Plants traits are under multiple and often conflicting selection pressures, and the production of secondary compounds is no exception (Adler 2000). The benefits of toxic nectar for plants, and subsequent selection for or against this plant trait, are therefore not
always clear. Plants utilize secondary metabolites in order to deter antagonists such as nectar robbers and thieves (Inouye 1980), florivores that consume flower petals and reproductive structures (McCall and Irwin 2006), and foliar herbivores (Slansky 1992)). On the other hand, if nectar secondary compounds become too high in concentration (e.g. if foliar and nectar secondary compound concentrations are correlated (Adler 2000, Manson et al. 2012)), they may deter pollinators and lead to a reduction in male or female plant fitness for animal-pollinated plants (Kessler et al. 2008, Adler and Irwin 2012, Manson et al. 2013). Although I did not directly measure the impacts of nectar toxins for plant fitness, two of my studies considered how nectar secondary compounds impact pollinator foraging behaviour in order to investigate this concept further (Chapter 2, 3).

My results from Chapters 2, 3 and 4 indicate that generalist *B. terrestris* will rarely avoid flowers with natural levels of nectar toxins. This of course implies that the fitness of plants with toxic nectar will not suffer from pollinator limitation if they are pollinated by similar generalist bee species. Other studies have suggested that pollinators may select for concentrations of nectar toxins below their deterrence thresholds (Wright et al. 2010, Wright et al. 2013); indeed, this phenomenon may have occurred in *R. ponticum*, at least in its native range (Chapter 2). Evidence for similar selection pressures in the invasive range of *R. ponticum* is also beginning to emerge. In parallel with my studies, Egan (2014) compared *R. ponticum* nectar GTX concentration, female plant fitness, pressure from herbivores and pollinator limitation in populations in the plant’s introduced and native range. He found a 2.5 fold decrease in nectar GTX concentration in the introduced range, which could not be explained by enemy release. This study suggests that pollinator mediated selection is occurring in the introduced range, because *R. ponticum* individuals with lower GTX concentrations are less pollinator limited and more fit. If invasive *R. ponticum* suffers from pollinator limitation (Egan 2014), plants may benefit from decreased GTX concentration if they can avail of pollination services from GTX intolerant pollinator species, such as *A. mellifera* and *A. carantonica* (Chapter 3). Because *A. mellifera* is an obligate flower-visiting social bee species it could provide substantial (and so far untapped, Chapter 4 and 5) pollination services for *R. ponticum* if nectar GTX concentrations were dramatically reduced (Chapter 3). My studies investigating pollinator foraging behaviour in response to nectar toxins only provide a piece of the puzzle required to understand the selection pressures influencing nectar chemistry. Nevertheless, they are important; understanding the significance that nectar toxins have on plant pollinator
interactions requires knowledge of how pollinators alter their behaviour in response to consumption of these compounds.

6.2.3 Community level impacts

Given that toxic nectar can directly and indirectly impact both plants and pollinators, it may also affect entire plant-pollinator communities. The species-level study in Chapter 3 provided interesting insights into the community level patterns of visitation observed in Chapters 4 and 5. The species-specific toxicity of GTX (Chapter 3) is likely responsible for the differences in visitation rates observed between bee taxa and *R. ponticum* (bumblebee visitation > solitary bee visitation > honeybee visitation, Chapter 4). Furthermore, the acute toxicity of *A. mellifera* towards nectar GTXs explains the almost complete absence of this bee species from *R. ponticum* dominated sites. *A. mellifera* commonly visits invasive plant species in other systems (Olesen et al. 2002, Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2008, Kaiser-Bunbury et al. 2011), and has even been implicated in facilitating plant invasion (Olesen et al. 2002). It is often a “super generalist” in invaded plant-pollinator networks (Olesen et al. 2002), and can alter the structure of these networks (Kaiser-Bunbury et al. 2011). The lack of *A. mellifera* at our sites, driven by the reaction of this generalist bee species to nectar toxins, could thus have a substantial impact on overall network structure.

Overall however, it is difficult to predict how nectar toxins might impact network structure. For example, in the case of *R. ponticum*, if nectar GTXs prevent certain species such as *A. mellifera* from participating in networks, there could be knock on effects for the abundance and visitation rates of other insect species. Perhaps the insects will have a broader diet breadth without *A. mellifera* dominating networks structure. Alternatively, if toxins in nectar have addictive or medicinal properties (Table 1.1), plants lacking this floral trait may be poorly connected in the network compared to plants containing toxic nectar. Although we did not observe compartmentalization in our networks (Chapters 4 and 5) such tendencies could lead to compartmentalization in larger, more complicated networks. The complexity of networks makes it difficult to predict how plant or insect traits may impact network structure, and this is certainly an area that requires further research (see section 6.3.2).

6.2.4 Role of toxic nectar in the invasion process

My work clearly demonstrates that toxic nectar can prevent some flower-visitors from visiting and potentially pollinating plant species (Chapters 3 and 4, Adler and Irwin 2005, Afik et al. 2007, Adler and Irwin 2012). As a result, I concluded that it is possible that toxic nectar may
present an additional barrier in the invasion process for introduced animal pollinated plants (Chapter 3); it could theoretically make it more difficult for plants to establish novel mutualistic relationships with pollinators in the introduced range (Richardson et al. 2000a). My community level studies, however, revealed that regardless of its toxic nectar, invasive *R. ponticum* is commonly visited by generalist insect species (Chapter 4, 5). Furthermore, the abundance, species richness and visitation rates to *R. ponticum* are the same in its native and introduced range (Chapter 5). At least when using *R. ponticum* as a model system, my results suggest that any barrier to invasion presented by toxic nectar will not be sufficient to prevent the establishment, naturalization and spread of an invasive plant species (Richardson et al. 2000a). It should however be noted that this may not always be the case; further studies in different systems are needed in order to test this hypothesis (see section 6.3.2). Furthermore, my work demonstrates that invasive plants with toxic nectar can have substantial negative impacts for native pollinators (Chapter 3). In order to protect pollinators and pollination services, it may behove conversationalists to be particularly wary of the introduction of alien plants with toxic nectar.

Hypotheses concerning toxic nectar

One of the main goals of researchers studying the chemical ecology of toxic nectar is to understand the occurrence of toxic or deterrent secondary compounds in floral nectar. Is there adaptive or functional significance, or do these chemicals end up in floral nectar as a consequence of defence of other plant tissues (Adler 2000)? As reviewed in Chapter 1 (Table 1.1), many hypotheses to explain the presence of toxins in floral nectar have been proposed and tested to varying degrees. Although my work does not produce a definitive answer to the question of why GTX is present in *R. ponticum* nectar, it is possible to review some of these hypotheses in a more meaningful way after reviewing my studies.

The pollinator fidelity and nectar robber hypotheses consider toxic nectar as a method of filtering flower visitors so that only the best pollinators visit (Table 1.1). Chapter 3 showed that there are species-specific effects of GTX for different bee species, however the pollination efficiency of these bees for *R. ponticum* is unknown. It is possible that *B. terrestris*, the only species we tested that did not exhibit observable negative impacts from GTX consumption, would be the best pollinator; it is the largest of the three species and *R. ponticum* flowers are very large and open, thus require a large bee to effectively pollinate (Stout et al. 2006). On the other hand, observational field data from Chapter 5 demonstrates that ants and pollen beetles, nectar and pollen robbers respectively, still feed on *R. ponticum*, regardless of the
presence of GTX in the nectar. There could, however, be detrimental impacts of this GTX on these visitors that were not observed in the field (e.g. negative consequences for immunity or fitness). The fact that antagonistic visitors were more common in the native range than the introduced range (Chapter 5) could indicate such detrimental impacts are occurring, at least in the introduced range. Concentrations of nectar toxins in this invasive species may be under conflicting selection pressures from pollinators and floral larcenists in the introduced range (Chapter 5).

We found no evidence to support the self-medication hypothesis (Table 1.1) using our study system. It does not appear that GTX impacts the levels of *Crithidia bombi* in *B. terrestris* at nectar realistic concentrations. However, additional studies could investigate other pathogens and parasites of *Bombus* species; it is possible that GTX may have medicinal properties against micro-organisms we did not test.

My work did not directly test the remaining hypotheses explaining the presence of toxins in floral nectar. In particular the antimicrobial, pharmacological, reduced nectar volume and drunken pollinator hypotheses (Table 1.1) require further examination in regards to our study system. Although we did not test the pleiotropy hypothesis directly either, I believe that it may be playing a significant role in our study system. Work from Egan et al. (2014) has demonstrated that floral concentrations of GTX I (the biologically active compound, Chapter 3) have decreased in the introduced range in comparison to the native range. This provides at least preliminary evidence of the conflict that occurs for selection for vs. against plant secondary chemistry in different plant parts. For example, if enemy release is occurring in the introduced range, perhaps less secondary compound is needed to protect some plant parts. If toxic nectar is a consequence of defence of other plant tissues, the reduction in concentration between the native and introduced ranges may occur as some pollinators select against *R. ponticum* plants with GTX I in their floral nectar. More work is certainly needed to fully understand why *R. ponticum* nectar contains high concentrations of these natural defensive plant compounds (see section 6.3.2).

### 6.3 Critical considerations

Throughout my studies, I used a variety of methods to investigate the impacts of plant invasion for native pollinators. My work was conducted at a variety of scales (individual, species and community, Chapters 2, 3, 4 and 5 respectively), incorporated manipulative (Chapters 2 and 3, Appendix A) and observational studies (Chapters 4 and 5), and was carried out both under
controlled laboratory (Chapters 2 and 3) and in field-realistic conditions (Chapters 4 and 5). With the aim of evaluating the relevance of the research presented here, in this section I will discuss some methodological limitations and considerations and provide suggestions for future research in this field.

6.3.1 Methodology

Among the first decisions that have to be made when conducting an assay on pollinator dietary preferences are whether study organisms will be free-flying (London-Shafir et al. 2003, Adler and Irwin 2005, Singaravelan et al. 2005, Adler and Irwin 2012) or restrained (Bitterman et al. 1983, Wright et al. 2010). Whether bees are free-flying or restrained may impact their nutritional requirements and subsequently their dietary preferences (Ayestaran et al. 2010). Free-flying individuals may be preferable because conditions during the experiment more closely estimate a natural situation. Hence, where possible I carried out my assays using free-flying bees contained in miniature flight cages (Chapter 2, partially Chapter 3 and Appendix A). This approach is well established, having been used in many assays to examine bee preference and behaviour (Detzel and Wink 1993, Singaravelan et al. 2006, Gegear et al. 2007, Pirk et al. 2009, Altaye et al. 2010). An exception was made in Chapter 3, where honeybees were harnessed while fed treatment solutions. This was necessary because free-flying honeybees would not feed on GTX solutions in isolation, and not enough solution was available to conduct the experiment with groups of bees. We accounted for this limitation by conducting an identical experiment with harnessed bumblebees (Chapter 3, Appendix C.2) in order to make a valid comparison between the two species.

The second design aspect I considered for my bioassays was whether each study should be a choice or non-choice assay. In each experiment, the appropriate type of assay was utilized to best answer the questions of interest. For example, the main objective of Chapter 2 and Appendix A was to determine the threshold of deterrence of *B. terrestris* for several chemicals found in floral nectar. This assay was therefore a paired choice assay, with each bee exposed to two equally concentrated sucrose solutions, one with and one without the chemical of interest. In contrast, assays in Chapter 3 were non-choice assays because we wanted to compare survival and consumption of individuals fed GTXs with control-fed individuals. It should however be noted that the foraging options available to pollinators (in other words the ecological context) can impact their dietary preferences and foraging behaviour (Gegear et al. 2007). Although my results from both types of assays were consistent (Chapters 2 and 3,
bumblebee results), care should be exerted when comparing results from choice and non-choice assays.

While Chapter 3 produced compelling results that are important from the point of view of pollinator conservation, the need for further research requires the conclusions be interpreted with some caution. I detected no negative impacts of the consumption of nectar GTXs on bumblebee survival. Furthermore, field studies support my conclusion that *R. ponticum* is an important forage resource for these long-season foraging bees (Stout 2007b, Stout 2007a, Dietzsch 2009). Nevertheless, recent studies on neonicotinoid pesticides demonstrate that even when lethal impacts are not realized, chemicals may have detrimental sublethal effects on pollinators (Gill et al. 2012, Henry et al. 2012, Whitehorn et al. 2012). Because of the difficulty of isolating GTX I from *R. ponticum* flowers and the inability to purchase it commercially, we did not have enough of the chemical to carry out assays investigating impacts on bumblebee fitness or physiology (Elliott et al. 2008, Manson and Thomson 2009). Although no apparent changes in bumblebee dietary preferences or behaviour were observed following GTX consumption (Chapters 2 and 3), a study investigating impacts on fitness would more conclusively rule out the possibility of negative sublethal impacts.

Both Chapters 4 and 5 utilized insect-flower interaction networks in order to examine the impact of invasive alien plants on pollinators. While the study of insect-flower (or "plant-pollinator") interaction networks is a useful way to carry out community level investigations, these techniques come with a host of methodological considerations, including:

- **Sampling method:** As mentioned in section 1.3.2, insect-flower interaction networks can be constructed using two main sampling methods: transect walks and timed observations (Gibson et al. 2011). There are benefits and drawbacks to both methods, but my network studies utilized timed observations (Morales and Aizen 2006, Olesen et al. 2008, Kaiser-Bunbury et al. 2011). This decision was made after careful consideration of the questions we wanted to answer, the resources available and especially the habitat characteristics of our sites. Transects are better suited in sites that are homogeneous (Memmott 1999, Lopezaraiza-Mikel et al. 2007, Alarcón et al. 2008), because a transect will be able to capture the diversity and relative abundance of flowering species at a continuous site (Gibson et al. 2011). *R. ponticum* grows in dense stands that shade out native flora (Cross 1975, Cross 1981); as a result, my sites were far from homogenous. Alternative flowering species were instead found at site
edges (by roads or streams), which were distinct from *R. ponticum* stands (Chapters 4 and 5). We used timed observations because they are more appropriate for heterogeneous habitats like forests (Gibson et al. 2011). Both methods introduce some level of sampling bias, but web asymmetry and evenness of marginal abundance distributions in particular should only be compared between networks that were constructed using the same methodology (Gibson et al. 2011). Neither of these metrics was used in my studies (Chapter 4 and 5).

- **Measuring floral abundance**: Gibson et al. (2011) demonstrate that when an independent measure of floral abundance is used to construct networks from timed observations, the ecological “realism” of this sampling method improves (Kaiser-Bunbury et al. 2009, Kaiser-Bunbury et al. 2011). Such networks can also be more readily compared to networks constructed using transect walks. In addition, I hypothesized that the relative abundance of an invasive plant is so high that it would be important to network structure (Kaiser-Bunbury et al. 2011, Stout and Casey 2014). As a result I included floral abundance measurements in the construction of my networks (Chapters 4 and 5). Measuring floral abundance in a heterogeneous site, however, proved to be a difficult task. Most studies carry out this measurement by recording the number of floral units of each plant species using random quadrats (Kaiser-Bunbury et al. 2009, Kaiser-Bunbury et al. 2011). The presence of dense *R. ponticum* stands at our site, as well as the height of these large shrubs (Cross 1975), made this method impossible. Instead, we used a stratified random sampling approach (Carvalheiro et al. 2008) based on the percent of the habitat that was covered by *R. ponticum* (Chapters 4 and 5). Most studies on plant invasion are carried out on more herbaceous species (Lopezaraiza-Mikel et al. 2007, Bartomeus et al. 2008, Bartomeus et al. 2010), and so do not encounter this kind of sampling issue. Shrubs, however, comprise a number of ecologically damaging invasive plant species (Richardson and Rejmanek 2011). My method for measuring floral abundance may be a useful tool in much needed future studies on the impacts of invasive shrub species.

- **What actually goes into the network?** The flowering times of the plant species at my sites, as well as a lack of time and resources, prevented me from sampling every plant species for exactly the same amount of time (Chapter 4 and 5). I accounted for this difference in effort by using ‘mean interaction frequencies’ (total number of visits observed /flowers observed/h observed * floral abundance of the plant species being
observed) to construct my networks (Carvalheiro et al. 2008, Kaiser-Bunbury et al. 2009, Kaiser-Bunbury et al. 2011). While this is a valid and commonly used method, it requires the use of non-integers in the network matrix. Non-integers can complicate the process of calculating some network parameters (e.g. measures of nestedness and asymmetry). If these questions are of particular concern, the transect method (and thus the use of integer values in networks) may be preferable.

There is little doubt that network analysis has improved our understanding of plant-pollinator community structure and can be a useful tool for the investigation of evolutionary and ecological relationships between plants and animals (Jordano 1987, Blüthgen et al. 2006, Blüthgen et al. 2007, Dormann et al. 2008, Petanidou et al. 2008, Dupont et al. 2009, Burkle and Alarcón 2011). Nevertheless, one must consider whether the many methodological issues and the time consuming nature of the sampling methods negate the usefulness of networks in considering more applied questions (Memmott 2009, Hegland et al. 2010). Used correctly, I believe insect-flower interaction networks can provide useful information on topics such as the impacts of plant invasion on community structure. Hegland et al. (2010) demonstrate that the majority of functionally important plant and insect species are captured with relatively little sampling effort. Furthermore, networks have already been used to understand issues such as habitat fragmentation (Steffan-Dewenter et al. 2006, Stanley and Stout 2014), species extinctions (Memmott et al. 2004), the conservation of rare plant species (Carvalheiro et al. 2008), and of course, the impacts of invasive alien species (Chapters 4 and 5, Memmott and Waser 2002, Olesen et al. 2002, Bartomeus et al. 2008, Bartomeus et al. 2010, Kaiser-Bunbury et al. 2011). As long as the proper considerations are taken when planning studies (Gibson et al. 2011) and interpreting results (Bosch et al. 2009, King et al. 2013), insect-flower interaction networks can continue to help answer critical applied questions about pollinators and pollination services.

Chapter 5 was unique from the rest of my studies because it utilized a biogeographical approach to compare aspects of insect-flower communities in *R. ponticum*’s native vs. its invasive range. Although invasion is a biogeographical phenomenon, a majority of studies of invasive species are carried out exclusively in the native range. Biogeographical comparisons are necessary to understand the success of invasive species in their introduced ranges, and future studies should utilize similar approaches (Hierro et al. 2005). Community level studies in particular are lacking a biogeographical approach; as I demonstrated in Chapter 5,
biogeographical comparisons can aid in answering fundamental questions about the impacts of introducing a novel plant to a plant-pollinator community.

Although over-arching conclusions about the impact of drivers of pollinator decline for overall pollinator abundance and diversity are useful from a policy-making and conservation perspective (Roberts et al. 2006), my work demonstrates the importance of studying individual species as well as entire communities. One of the strengths of this thesis is that it studied a potential driver of pollinator decline at multiple scales, and was therefore able to discern both species and community level effects. I carried out studies considering the responses of individuals of the same pollinator species (Chapter 2), multiple pollinating species (Chapter 3), entire pollinating communities (Chapter 4), and finally, compared pollinator communities on a biogeographical scale (Chapter 5). Without each of these levels, important messages for conservation (such as the potential mitigating effects of *R. ponticum* invasion towards other drivers of declines for GTX tolerant bumblebees, Chapters 2 and 3) would not have been understood. Differing responses of pollinator taxa have also been demonstrated by recent studies on additional drivers of pollinator decline, such as anthropogenic disturbance (Winfree et al. 2009), habitat loss (Steffan-Dewenter et al. 2006) and exposure to agrochemicals (Goulson 2013). Future studies on drivers of pollinator decline may therefore benefit from a similar multi-scale approach.

6.3.2 Directions for future research

While my research has substantially aided understanding of the impacts of plant invasion for native pollinators, there are several further considerations that should be explored.

With regards to *R. ponticum* specifically, it would be interesting to understand the mechanism that leads to species-specific toxicity of GTX for bees. It may be a result of differences in the structure of sodium channels between species, but molecular and genetic techniques would be required to verify this hypothesis. From a conservation and management perspective, it would also be pertinent to test more species of pollinators for lethal and sublethal responses to nectar GTXs. Very little is known about the impacts of nectar GTXs on butterfly species in particular, and information on additional solitary bee species would also be valuable.

It would also be useful to approach the subject of toxic nectar in invasive plant species from a broader perspective. For example, an extensive geographic and phylogenetic survey of the nectar of invasive plants could lend insight into what (if any) nectar traits impact plant invasion (Milbau and Stout 2008). Such a survey could be similar to the studies of the nectar of native
plants carried out by Baker and Baker in the 1970's. Ultimately it could result in a database with a plethora of information on the nectar of invasive plants, including sugar, amino acid and secondary metabolite concentrations and rate of production. This resource could be useful in helping predict which invasive plants will most impact native pollinators. It could also be used to investigate whether nectar toxins can provide a barrier to invasion for some plant species.

As mentioned above, very few studies have investigated the impact of nectar secondary compounds on pollinator fitness (but see Elliott et al. 2008, Manson and Thomson 2009). In regards to toxic nectar, this is the biggest gap in the literature to date. Microcolony studies using bumblebees would be a useful approach to tackle this question because 1.) bumblebees have an annual life cycle so they have a clear end to their reproductive cycle, 2.) microcolonies, as opposed to full colonies, make the experiments more feasible logistically, 3.) bumblebees are important pollinators ecologically and economically that often visit plants with secondary compounds in their nectar (Adler and Irwin 2005, Cook et al. 2013, Manson et al. 2013). Given the negative impacts of secondary compounds on the fitness of foliar herbivores (Slansky 1992), one might expect a similar reaction from pollinators; however because of the reduced concentrations usually found in floral nectar this may not be the case (Manson et al. 2012).

My thesis focused on how invasive alien plants impact the forage resources available to pollinators, but impacts on additional resources could also be important. There is practically no information on how invasive plants (and in particular large shrubs like *R. ponticum*) impact nest site availability (but see Edwards and Jenner 2005, McKinney and Goodell 2010). In addition, impacts on larval food resources are unknown, but can be important for certain pollinating species like Lepidopterans (Graves and Shapiro 2003) and Dipterans. Studies investigating these additional resources may provide useful information that could help reconcile community level and species-specific responses of pollinators to invasion by introduced plants.

One area of network analysis that still remains unclear is the ecological meaning associated with some of the parameters used to describe insect-flower interaction networks. A better understanding of which network properties are beneficial for conservation would make the interpretation of results much more meaningful (Tylianakis et al. 2010). Work considering which parameters confer stability and robustness to networks should therefore be prioritized.

With regards to the main question this thesis was trying to address, what are the impacts of invasive plants for native pollinators, a considerable amount of work remains. Due to the
variation in the responses of pollinators to biological invasions (Goodell 2003, Graves and Shapiro 2003, de Groot et al. 2007, Moron et al. 2009), even when considering the same invasive plant species (Chapter 3), there is a great need for more studies on this subject. The progression of this field of study seems similar to that of studies investigating the impact of plant invasion on native plant pollination. At first, very few studies were carried out, and much of the variation in responses was attributed to the variety of traits of native and invasive plants that impact reproduction (e.g. Dietzsch et al. 2011). Eventually though, enough studies were performed to allow for generalizations through meta-analysis, and it was found that invasive plants most often have negative impacts on native plant pollination (Morales and Traveset 2009). Due to the variation in the responses of pollinators to plant invasion, more studies are needed before such a meta-analysis could generate similar meaningful generalizations.

In ecology, more attention is often given to model systems that are convenient to study or are of economic, agronomic, or medical value (Schumann 1991). The European honeybee, *A. mellifera* is well studied compared to other bee species (Potts et al. 2010a), due to its use as a managed pollinator for certain crop species, the value associated with hive products (honey and wax), and its use as a model species in laboratory experiments. Concerns have been voiced that the majority of funding and research efforts into pollination and pollinator declines are allocated to studies of this one species, to the detriment of our understanding of patterns in potentially more important wild pollinators (Morse and Corbet 1991, Aebi et al. 2012, Ollerton et al. 2012a). On the other hand, the growing recognition of the importance of wild pollinators for the pollination of crops and maintenance of biodiversity (Klein et al. 2007, Winfree et al. 2008, Breeze et al. 2011, Garibaldi et al. 2013) could lead to a scarcity in the number of studies of certain drivers of decline focusing on managed honeybees (Winfree et al. 2009). Finally, many studies investigating bumblebees have focused on commercially available species (Manson and Thomson 2009, Manson et al. 2010, Gill et al. 2012, Whitehorn et al. 2012), which tend to be broad generalists with relatively large colonies and may therefore be less susceptible to certain drivers of decline. Any taxonomic bias in the research efforts examining each of the five main drivers of pollinator decline (Potts et al. 2010a) could be damaging to conservation efforts. As this thesis has demonstrated, mitigation of a pressure (e.g. removal of an invasive species) may be beneficial to one group of pollinators, but not to another. Furthermore, conservationists often use quantitative reviews or meta-analyses in order to create management plans (Roberts et al. 2006). Studies on drivers of pollinator decline that focus on a variety of taxa or guilds of pollinators allow for taxonomic groups to be
taken into account in meta-analyses (Winfree et al. 2009, Montero-Castaño and Vila 2012). This will subsequently allow for proper management of target species or groups. It is therefore critical that we determine if a taxonomic bias exists in the studies of each of the five main drivers of pollinator declines, in order to correct the bias and best understand how to manage our pollinator populations. A meta-analytical approach could be a useful method for tackling this issue. It is also important that studies on all drivers of pollinator decline, including invasive alien species, consider the impacts of multiple interacting global change pressures. As this thesis demonstrated, such interactions can have both antagonistic and synergistic effects that could be crucial for pollinator conservation.

6.4 Concluding remarks

The critical ecosystem service of pollination is threatened by the worldwide decline of pollinators (Allen-Wardell et al. 1998, Kearns et al. 1998). There is a need for further research on drivers of pollinator decline in order to halt this loss of biodiversity and ensure proper functioning of our ecosystems. Fortunately, the biased view of invasive alien species as always having negative impacts on flora and fauna is giving way to a more comprehensive, evidenced based view (Rodriguez 2006). It is very difficult to make generalizations about the impacts of plant invasion for native pollinators because the direction of impacts is modulated by plant and pollinator traits, and community composition. Invasive alien plants are therefore likely to play different roles in the pollinator crisis depending on what taxa are being considered. One such trait that had not previously been considered in the context of plant invasion was the presence of secondary compounds in floral nectar. When taken as a whole, my work suggests a framework for the consideration of toxic nectar in the invasion process.

As a result of the above-mentioned variation in plant and pollinator traits, before management decisions are made regarding introduced plants, researchers should evaluate impacts for pollinators using studies at a variety of scales, from individuals, to species, to communities. This is true not only for studies of invasive alien species, but of all drivers of pollinator decline. Finally, as the science in this field advances, it is becoming clear that multiple interacting drivers of decline must be taken into account if we are to protect our valuable pollinators and the services they provide (Gonzalez-Varo et al. 2013, Vanbergen and Insect Pollinators Initiative 2013).
Appendices
Appendix A: Bumblebee preference for sucrose solutions which contain low concentrations of neonicotinoid insecticide

A.1 Abstract

Neonicotinoids, a class of widely used systemic insecticides, have been found in the nectar and pollen of treated plants, and thus may negatively impact beneficial pollinators. Levels of exposure to these insecticides partially depends upon how often pollinators choose to feed on contaminated crops, but little is known about how neonicotinoids impact the dietary preferences of insects. This study used simple paired choice experiments to investigate whether field-relevant concentrations of the neonicotinoids imidacloprid and clothianidin impact the dietary preference of Bombus terrestris workers under controlled conditions. Bumblebees preferred solutions containing concentrations of imidacloprid as low as 1 nM over equally concentrated sucrose controls. This preference persisted for 10 nM and 100 nM solutions, but reversed when concentrations were increased to 1000 nM. Bees exhibited no preference for solutions containing the neonicotinoid clothianidin at nectar-realistic concentrations, but were similarly deterred by 1000 nM solutions. Finally, bumblebees exhibited no preferences in response to similar concentrations of nicotine, a natural plant secondary compound with the same mode of action as neonicotinoid pesticides. Bumblebee preference for solutions containing nectar-realistic concentrations of imidacloprid indicates that their natural exposure levels may be higher than previously thought. Further field based, electrophysiological, and behavioural studies are required to test our hypothesis that some neonicotinoids may impose dependence or addiction effects on bees.

Data from this chapter are included in a manuscript written in collaboration with members of G.A. Wright’s research group, submitted to Nature in 2015. I am a co-first author on this manuscript.
A.2 Introduction

Chronic exposure to pesticides is considered a main factor contributing to the worldwide decline of pollinators, and in particular bees (Goulson et al. 2008, Brittain and Potts 2011, Gonzalez-Varo et al. 2013, Vanbergen and Insect Pollinators Initiative 2013). Neonicotinoids are currently the most widely used class of pesticides in the world, licensed for use on arable crops in over 120 countries (Elbert et al. 2008, Krupke et al. 2012). Neonicotinoid insecticides are particularly advantageous for pest control because they are systemic; when applied as a seed dressing or through foliar sprays, the active ingredient is easily absorbed by plants through roots or leaves and distributed throughout the rest of the tissues, providing prophylactic protection against herbivores (Goulson 2013). Due to their systemic nature, neonicotinoids are also found in plant nectar and pollen (Schafer 2004). Thus beneficial pollinators foraging for these resources on treated mass-flowering crops, such as oil seed rape and sunflower (Fell 1986, Diekötter et al. 2010), frequently encounter neonicotinoid insecticides.

Given recent concerns over the well-documented decline of pollinators (Biesmeijer et al. 2006, Natural Research Council 2007, Potts et al. 2010b, Cameron et al. 2011), studies investigating the impacts of neonicotinoid exposure on bees have increased. Laboratory and semi-field toxicity tests indicate that concentrations of neonicotinoids found in the nectar and pollen of seed-treated crops are not directly lethal for bees (Cresswell 2011, reviewed in Goulson 2013). However, exposure to these concentrations can have sublethal impacts for both honey and bumblebees, such as decreases in learning, foraging efficiency and homing capabilities (Tomizawa et al. 2000, Ramirez-Romero et al. 2005, Gill et al. 2012, Henry et al. 2012). For bumblebees, the evidence is particularly compelling that neonicotinoids could have long-term colony and population-level impacts. Consumption of nectar realistic doses of these insecticides reduces bumblebee foraging efficiency (Gill et al. 2012, Feltham et al. 2014) and subsequently decreases the rate of nest growth, brood production and queen production (Laycock et al. 2012, Whitehorn et al. 2012).

The levels of neonicotinoids bees are exposed to in field-realistic conditions will ultimately determine the impact of these chemicals on pollinator populations. To date, however, studies have used experimentally exposed bees where the bees have no way to avoid exposure to neonicotinoids. In natural conditions, bees will choose from a variety of floral resources, including wildflowers growing in field margins and semi-natural habitats, as well as bursts of mass-flowering crops that are likely to contain neonicotinoids (Power and Stout 2011, Stanley
and Stout 2013). If neonicotinoids influence the dietary preferences of bees, field realistic exposure levels could vary considerably. For example, natural plant secondary compounds—also commonly found in floral nectar and pollen (Adler 2000)—can have a range of dose-dependent impacts on bee dietary preferences (Detzel and Wink 1993, Hagler and Buchmann 1993). Honeybees were deterred by nectar-relevant concentrations of anabasine, but demonstrated a preference for low concentrations of the psychoactive alkaloids nicotine and caffeine (compared to controls) (Singaravelan et al. 2005). In addition, a recent study showed that pan traps containing field-realistic concentrations of the neonicotinoid imidacloprid repelled pollinating flies and beetles (Easton and Goulson 2013), although impacts on feeding responses were not investigated. Studies suggest that honeybees may avoid imidacloprid in nectar, but at insecticide concentrations higher than those naturally encountered in the nectar of treated crops (Ramirez-Romero et al. 2005).

We carried out simple paired choice experiments to investigate the impact of field-realistic concentrations of neonicotinoids on bumblebee dietary preferences. We asked whether Bombus terrestris workers demonstrated a preference for or against sucrose solutions containing nectar-realistic concentrations of imidacloprid and clothianidin when offered an equally rewarding control solution. The information about bumblebee feeding preferences provided by our study is a critical first step towards understanding the potential exposure levels of bees to these toxic neonicotinoids.

A.3 Materials and Methods

Assays were run using two of the most widely used neonicotinoids, imidacloprid and clothianidin (Goulson 2013), and one natural plant secondary compound, nicotine (Sigma-Aldrich, Dorset, UK). Nicotine was included to allow for a comparison between the neonicotinoids and a natural plant chemical with a similar mode of action; all three compounds are nicotinic acetylcholine receptor (nAChRs) agonists (Tomizawa et al. 2000).

Bombus terrestris dalmatinus workers from at least three colonies (Unichem Ltd, Co. Dublin, Irish distributor for Koppert) were used for each assay (total 11 colonies). Colonies were maintained at 25-30 °C in 24 h darkness and fed commercial pollen and Biogluc (Agralan Ltd, Swindon) bee food ad libitum.

We examined the influence of each compound on the dietary preferences of bumblebee workers using a 24 h paired choice assay. Individual bees were offered two sucrose solutions, one with and one without the compound at a range of nectar relevant concentrations. Due to
the controversy concerning the concentrations of neonicotinoids in the nectar of mass-flowing crops (EFSA 2012), we used a variety of low doses ranging from 1 nM – 1000 nM. Sucrose solutions were 0.5 M to represent a concentration commonly found in bee-pollinated flowers (Baker and Baker 1975) and were made by mixing grade II sucrose (Sigma-Aldrich) with deionised water. Serial dilutions were carried out with imidacloprid, clothianidin and nicotine in order to create test solutions.

Bees were removed from each colony, weighed and measured and transferred to individual 650 ml plastic containers (160x110x45mm) for the 24 h experimental period. Containers were fitted with three 3 ml feeding tubes, inserted horizontally (Plate 4). Feeding tubes had four 2 mm holes so bees could alight on the tubes and feed from the openings. Bees were given a choice between two sucrose solutions: a 0.5 M sucrose solution (internal control) and the same solution containing a concentration of the toxin. Deionised water was provided in the third tube, but was rarely visited by individuals and excluded from analyses. Tubes were weighed prior to exposure to a bee and 24 h after and the amount of solution consumed from each was calculated. Experiments took place in growth cabinets at 28°C, 60% relative humidity and 24 h darkness in order to mimic nest conditions (Heinrich 2004). Eight external controls (identical setups containing no bees) were set up for each concentration of each compound in order to control for evaporation. Data from individual bumblebees were utilized only if the bee was still alive at the end of the 24 h test period, except for bees in the 1000 nM treatment for imidacloprid and clothianidin; because of the high toxicity of this dose, all bees were included in the analysis. Experiments were replicated until at least 40 and up to 65 bees were exposed to each concentration combination of each compound (for further details on experimental design see Tiedeken et al. 2014).

Plate 4. Experimental setup for paired choice bioassays. Feeders were inserted horizontally so bees could feed ad lib on either of the two treatment solutions.
Data were analyzed using generalized linear models (GLMs) with repeated measures. Concentration, solution type (presence/absence of the toxin) and their interaction were included in the model and a least significant difference (LSD) post hoc comparison was used for all pairwise comparisons. All analyses were carried out using the statistical package SPSS Statistics, version 20 (IBM).

### A.4 Results

Imidacloprid had a significant impact on consumption, but the direction of the impact depended upon the concentration being tested (Table A.1). Bumblebees demonstrated a significant preference for solutions containing imidacloprid at 1 nM, 10 nM and 100 nM concentrations when compared to the sucrose control ($P = 0.042$, 0.016 and 0.019 respectively, Figure A.1a). Conversely, bumblebees avoided the 1000 nM imidacloprid solution and fed preferentially from the sucrose control ($P = 0.009$). Clothianidin also significantly impacted consumption, (Table A.1) but bees did not prefer solutions containing this compound at any concentration (Figure A.1b). Similar to imidacloprid, bees preferred the sucrose control over the 1000 nM clothianidin solution ($P = 0.017$), and bees in this treatment group consumed significantly less solution overall. Finally, unlike either of the neonicotinoids, nicotine had no impact on consumption or dietary preferences at any of the concentrations we tested (Table A.1, Figure A.1c).

### Table A.1

Wald $\chi^2$ test of model effects of imidacloprid, clothianidin and nicotine on *B. terrestris* feeding response. Concentration, solution type (presence/absence of the compound), and their interaction were included in the model and a least significant difference (LSD) post hoc comparison was used for all pairwise comparisons.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Concentration</th>
<th>Solution</th>
<th>Solution*Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>92.32</td>
<td>6.90</td>
<td>20.57</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.001^{***}$</td>
<td>$P = 0.009^{**}$</td>
<td>$P &lt; 0.001^{***}$</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>453.95</td>
<td>0.032</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.001^{***}$</td>
<td>$P = 0.858$</td>
<td>$P = 0.472$</td>
</tr>
<tr>
<td>Nicotine</td>
<td>4.45</td>
<td>1.14</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>$P = 0.217$</td>
<td>$P = 0.285$</td>
<td>$P = 0.771$</td>
</tr>
</tbody>
</table>
Figure A.1  *B. terrestris* mean (± SE) consumption (g), controlled for evaporation of 0.5 M sucrose solution with (dark grey bars) or without (light grey bars) a. imidacloprid, b. clothianidin, or c. nicotine. Asterisks indicate significant differences between consumption of two solutions at a given concentration according to least significant difference (LSD) post hoc comparisons (*P < 0.05, **P < 0.01).
A.5 Discussion

There is no doubt that neonicotinoids have the potential to harm beneficial pollinators (Tomizawa and Casida 2005, Goulson 2013), however, the severity of the impacts of these chemicals depends upon realistic exposure levels (EFSA 2012). Whether or not bees can detect and avoid nectar and pollen containing neonicotinoids will ultimately influence their foraging decisions and subsequent exposure. Our results indicate that bees are not likely to avoid forage resources contaminated with neonicotinoids in a natural setting, and may even exhibit a dietary preference for certain compounds. If such a preference persists, field level exposure of bees to some neonicotinoids may be even higher than previously estimated.

The natural plant compound nicotine is structurally similar to neonicotinoids (Tomizawa et al. 2000, Tomizawa and Casida 2005), and honeybees have been found to prefer nicotine-containing solutions over sucrose controls (Singaravelan et al. 2005). Nicotine likely did not elicit a feeding response from *B. terrestris* workers in our assays because of the low concentrations we used. Nectar realistic concentrations of nicotine are several orders of magnitude higher than concentrations of neonicotinoids in nectar (Detzel and Wink 1993, Tadmor-Melamed et al. 2004). Indeed, in similar paired choice assays using higher doses of nicotine, the minimum concentration required to deter *B. terrestris* was 0.1 mM, nearly 100 times higher than the highest concentration used in our assays (Tiedeken et al. 2014). Regardless of the concentration, however, no evidence has been found that bumblebees prefer solutions containing nicotine over equally rewarding controls (Tiedeken et al. 2014).

In contrast to nicotine, imidacloprid and clothianidin impacted the feeding preferences of bumblebees in our assays. The highest concentration of both compounds, 1000 nM, was deterrent and led to reduced overall consumption compared with the other treatments. This concentration, however, is likely to be higher than levels normally encountered by foraging individuals (EFSA 2012). The average concentration of neonicotinoids in the nectar of seed dressed crops, calculated from 20 studies, is approximately 1.9 ± 0.5 ppb, or approximately 7 nM (EFSA 2012, reviewed in Goulson 2013). While clothianidin did not impact preference at these low concentrations, imidacloprid solutions were preferred at concentrations as low as 1 nM, well below the average. Only one other study demonstrates biological activity of neonicotinoids at concentrations below the limits of laboratory detection (Easton and Goulson 2013), and in combination with our findings this indicates that the sensory systems of invertebrates are very sensitive to these toxic insecticides.
Our finding, that bumblebees are unlikely to avoid, and may even prefer food containing neonicotinoids, is consistent with field observations. Bumblebee abundance and density have been shown to increase in landscapes with mass-flowering crops that are likely to be treated with neonicotinoid insecticides, such as oilseed rape (Westphal et al. 2003, Stanley and Stout 2013). This increase in bumblebee abundance is usually attributed to the pulse of sugar rich nectar and pollen provided by mass-flowering crops (Hanley et al. 2011), but our study indicates that preference for neonicotinoid-containing nectar may also attract bumblebees. In addition, the increased resource availability provided by mass-flowering crops is often thought to play an important role in bumblebee conservation (Westphal et al. 2003, Herrmann et al. 2007). Decreased colony fitness resulting from sublethal effects of neonicotinoid consumption (Whitehorn et al. 2012) may outweigh the benefits of increased resource availability, and could have severe consequences for bumblebee populations. Indeed, this mechanism may explain why exposure to landscapes with mass flowering oilseed rape has been found to improve early colony growth, but not sexual reproduction, of bumblebees (Westphal et al. 2009).

Our data provide compelling evidence that some neonicotinoids at nectar-realistic concentrations may elicit a feeding preference for B. terrestris workers, but little is known about impacts on other taxa. A recent study found that low concentrations of imidacloprid in yellow pan traps repelled Diptera and Coleoptera, but increased the catches of spiders (Easton and Goulson 2013). Because no reward was offered to insects in this study it is difficult to compare our results; indeed the deterrent effects of some nectar alkaloids for bumblebees can be offset by high sugar rewards (Gegear et al. 2007). Results from another study indicate that honeybees may be repelled by imidacloprid in nectar, but at concentrations much higher than encountered in nectar from seed dressed crops (Ramirez-Romero et al. 2005). It is also important to consider more realistic foraging situations, where bumblebees have multiple food sources to choose from and many other cues influencing forage decisions (e.g. accessibility of floral rewards, abundance of flowers, Goulson 2010). Our experiments should be supplemented by studies carried out in a field setting and for longer time periods, to determine whether the preference we observed for imidacloprid is consistent over time and space. Finally, our experiment only demonstrates impacts of neonicotinoids on feeding response; it is possible that bumblebees are able to detect these compounds at lower concentrations, but their preferences are not impacted. Electrophysiological experiments
would provide more information on the concentrations at which bees can truly “taste” or detect neonicotinoids.

A.5.1 Potential implications of our study

In recent years, authors have begun to speculate on whether naturally occurring nectar secondary compounds, such as nicotine and caffeine, may have psychoactive properties for pollinators similar to those found in the mammalian literature (Singaravelan et al. 2005). There is some evidence that nicotine in particular induces addictive behaviours in invertebrates such as the nematode Caenorhabditis elegans (Schafer 2004) and insects such as Drosophila (Bainton et al. 2000). Neonicotinoids are structurally similar to nicotinoids (e.g. nicotine), (Tomizawa and Casida 2005); perhaps, at low concentrations, they could have positive reinforcing, or rewarding, properties for some insects. We hypothesize that some neonicotinoids have psychoactive properties that cause dependence or addiction effects at the low doses pollinators experience in the nectar of treated flowering crops. Characterizing and demonstrating addiction in insects is challenging, but given our findings on the impacts of imidacloprid on bumblebee dietary preferences, this is a topic that demands further investigation.

A.5.2 Conclusions

Our work indicates that bumblebee workers do not avoid food containing low concentrations of neonicotinoids even when an uncontaminated, equally rewarding alternative is available. Furthermore, we demonstrate that bumblebees exhibit a preference for solutions containing low, nectar-realistic concentrations of imidacloprid, at least in a controlled laboratory setting. This finding is relevant to the current debate on the impacts of neonicotinoids to pollinators because it indicates pollinators will freely forage on contaminated crops. Studies demonstrating harmful impacts of neonicotinoids to bees have been criticized for exposing test organisms to concentrations higher than they would experience in a field realistic scenario (EFSA 2012). We demonstrate that in reality, levels of exposure for bumblebees may be underestimated. The impact of biologically active chemicals found in the nectar and pollen of beneficial pollinators is of great concern, and impacts on dietary preference should be given more attention.
Appendix B: Previous experimental or observational studies of the impacts of plant invasion on native pollinators.

Table B.1 Experimental or observational studies of the impacts of plant invasion on native pollinators. Response variables often, but not always, included abundance or species richness of entire pollinator community. Pollinators were observed using one of three study designs: invaded vs. uninvaded, invaded vs. invasive removed/ natural habitat restored, or invasive vs. native co-generic plants.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Positive or negative impact of invasive plant species on pollinators</th>
<th>Invasive plant species</th>
<th>Response variable</th>
<th>Study design</th>
<th>Country where study took place</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Shapiro 2002)</td>
<td>POSITIVE: butterflies rely on introduced, naturalized flora</td>
<td>Variety of introduced plant species in California</td>
<td>Behaviour and feeding preferences of butterfly fauna of Davis, California</td>
<td>Essentially a review based on observations by the author.</td>
<td>United States, California</td>
</tr>
<tr>
<td>(Novotny et al. 2003)</td>
<td>POSITIVE: higher median species richness of Lepidoptera larvae on invasive plants when compared to native plants</td>
<td><em>Piper aduncum</em> and <em>P. umbellatum</em></td>
<td>Spp. richness of Lepidoptera larvae</td>
<td>Compared two invasive plant spp. with that of 69 native plants, including one congeneric species.</td>
<td>Indonesia, Papua New Guinea</td>
</tr>
<tr>
<td>(Goodell 2003)</td>
<td>MIXED: NEGATIVE for overall species richness, but POSITIVE for bumble and honeybees</td>
<td>Purple loosestrife, <em>Lythrum salicaria</em></td>
<td>Bee taxa, bumble and honeybees examined separately</td>
<td>Invaded vs. uninvaded calcareous fens in NJ</td>
<td>United States, California</td>
</tr>
<tr>
<td>(Graves and Shapiro 2003)</td>
<td>MIXED: POSITIVE for butterfly fauna that feed on invasive plants as larvae or adults, negative when the invasive plants are toxic to larvae</td>
<td>Variety of introduced plant species in California</td>
<td>Butterfly fauna of California</td>
<td>A meta-analysis compiling data on invasive plants in the region, and their use by butterfly fauna of CA.</td>
<td>United States, California</td>
</tr>
<tr>
<td>(Ernst and Cappuccino 2005)</td>
<td>NEGATIVE: lower arthropod diversity and abundance on invasive plant species, IAP supported few pollinator spp.</td>
<td><em>Vincetoxicum rossicum</em></td>
<td>Pollinator abundance/richness. (Looked at all arthropods).</td>
<td>Compared invasive plant species with three native old field plants.</td>
<td>Canada, Ottawa/Ukraine and Russia</td>
</tr>
<tr>
<td>(Tepedino et al. 2008)</td>
<td>POSITIVE: found no difference in bee species richness between natives and invasives and invasive plants had on average twice as many bee spp. visit as natives, though all but one were generalists.</td>
<td><em>Tamarix spp.</em>, <em>Melilotus albus</em>, <em>Melilotus officinalis</em></td>
<td>Native bee abundance and species richness</td>
<td>Three invasive plant species compared with seven concurrently flowering native species.</td>
<td>United States, Utah</td>
</tr>
<tr>
<td>(de Groot et al. 2007)</td>
<td>MIXED: NEGATIVE for ab., sp. rich. and div. of butterflies, and for hoverflies before flowering of the invasive, but POSITIVE for hoverflies during flowering.</td>
<td><em>Solidago canadensis</em></td>
<td>Abundance, species richness and diversity of butterflies, hoverflies and carabid beetles</td>
<td>Invaded vs. uninvaded herbaceous semi-natural habitats</td>
<td>Europe, Slovenia</td>
</tr>
<tr>
<td>(Moroň et al. 2009)</td>
<td>NEGATIVE: Negative effect on pollinator abundance, diversity and species richness</td>
<td><em>Solidago canadensis</em>, <em>Solidago gigantea</em></td>
<td>Entire wild pollinator communities, including wild bees, hoverflies and butterflies</td>
<td>Invaded vs. uninvaded wet meadows</td>
<td>Europe, Poland</td>
</tr>
<tr>
<td>(Nienhuis and Stout 2009)</td>
<td>NEUTRAL: No impact of invasion on pollinator abundance, bumblebee abundance, or functional insect diversity</td>
<td><em>Impatiens glandulifera</em></td>
<td>Pollinator abundance, looked specifically at bumblebees, solitary bees and specialist vs. generalist bees.</td>
<td>Invaded, uninvaded and experimentally removed sites</td>
<td>Europe, Ireland</td>
</tr>
<tr>
<td>(Jakobsson et al. 2009)</td>
<td>POSITIVE: at low densities, the invasive species attracts pollinators.</td>
<td><em>Oxalis pes-caprae</em></td>
<td>Measured visits of native pollinator taxa to a native flowering plant</td>
<td>Invaded, uninvaded, and partially invaded</td>
<td>Europe, Spain</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Positive/Negative</td>
<td>Description</td>
<td>Methodology</td>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Dietzsch 2009</td>
<td>POSITIVE: R. ponticum provides a suitable food resource for Bombus spp. and invaded sites have more bumblebee colonies than uninvaded sites.</td>
<td>Rhododendron ponticum Looked at colony density of two Bombus species, (lucorum and pascuorum), and carried out lab bioassays with R. ponticum food sources.</td>
<td>Invaded vs. uninvaded sites for colony density</td>
<td>Europe, Ireland</td>
<td></td>
</tr>
<tr>
<td>McKinney and Goodell 2010</td>
<td>NEGATIVE: invasive plant increased understory shade reducing pollinator visitation</td>
<td>Lonicera maackii Looked at pollinator visitation to a native plant</td>
<td>Removal of invasive biomass and of flowers vs. control unremoved</td>
<td>United States, New Jersey</td>
<td></td>
</tr>
<tr>
<td>Hanula and Horn 2011</td>
<td>NEGATIVE: Removing this invasive shrub INCREASES native bee diversity and abundance</td>
<td>Ligustrum sinense Looked at bee species abundance diversity and community similarity.</td>
<td>Invaded vs. removal sites; had two types of removal, an invaded control, and an uninvaded treatment called “desired future conditions.”</td>
<td>United States, Georgia</td>
<td></td>
</tr>
<tr>
<td>Williams et al. 2011</td>
<td>POSITIVE: Native bees are utilizing, but not preferring, resources provided by alien invasive plants, particularly in disturbed habitats.</td>
<td>Many invasive species, study was carried out in CA and NJ Looked at bee foraging preferences and behaviour, and also abundance and richness.</td>
<td>Sampled bees and vegetation at sites with varying levels of disturbance</td>
<td>United States, New Jersey</td>
<td></td>
</tr>
<tr>
<td>Baskett et al. 2011</td>
<td>MIXED: POSITIVE: abundances of pollinator taxa were 5x’s higher in invaded sites than uninvaded sites. BUT NEGATIVE: plot-level removal treatments increased pollinator visits to a threatened plant species.</td>
<td>Gypsophila paniculata Centaurea maculosa Looked at all pollinator taxa, richness and abundance</td>
<td>Invaded vs. uninvaded and experimentally removed sites.</td>
<td>United States, Michigan</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C: Accompanying Chapter 3

C.1 Extraction and isolation of grayanotoxins from *R. ponticum* flowers

Dried *R. ponticum* flowers (100g) were extracted in 2l of 50% Methanol for 24h. Methanol was removed under vacuum in a rotary evaporator and the water extract partitioned once with hexane (500ml) followed by chloroform (500ml) six times and evaporated under vacuum. The combined weight of dried partitioned extract partitioned into chloroform was ~300mg. A C18 isolute column (internal diameter 20mm), adsorbent volume was 70ml and column solvent volume approximately 20ml was conditioned in methanol and then increasing water to a starting condition of 2% methanol. The dried chloroform partition was solubilised in 5ml 2%MeOH and applied to the top of the column and drawn onto the column under low vacuum. The extract was eluted with increasing concentration of methanol and fractions analysed by LC-MS. Grayanotoxin 1 eluted with 40% methanol and verified by comparison with a known standard as previously reported (Tiedeken et al. 2014). The extraction and isolation was repeated 14 times (total 1.4 kg *R. ponticum* flowers) to yield ~400 mg grayanotoxin. The isolated compound crystallized when drying under nitrogen from methanol.

C.2 Bumblebee harnessed assay

Two hundred and fifty bees were removed from three commercial colonies and placed into plastic vials. Bees were chilled on ice and weighed and measured (body length, thorax and abdomen width) and harnessed. Harnesses consisted of 2 ml Eppendorf tubes cut at an angle 1 cm below the tip, so that the bee’s head could be pulled through. The bee’s proboscis and neck were unobstructed and cotton wool was put in the bottom of the tube to keep the individual in place. Bees were fed up to 5 x 5 μL 50% Apinvernt solution two hours after harnessing. Because bumblebees are much less likely to feed when restrained, individuals that did not feed on Apinvent were not discarded. Bees were kept at 28°C, 60% RH and 0 light in growth chambers (Heinrich 2004). The following day, 50 bees were randomly assigned to the five treatments (see Table 3.1) and fed their treatment solutions, 5 x 5 μL. Only bees that fed while harnessed (35%) were included in the analyses. Bees were observed for 6 h for survival.
Table C.1. Four *R. ponticum* populations from which plant tissues were collected. Tissue type refers to the category of tissue collected in a given year: nectar, from which sugar content and grayanotoxin concentration were determined, and flowers, from which grayanotoxin was isolated for use in artificial nectar. Populations were located in the southeast and midlands in Ireland.

<table>
<thead>
<tr>
<th>Site name</th>
<th>County</th>
<th>Coordinates</th>
<th>Collection year</th>
<th>Tissue collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dunran</td>
<td>Wicklow</td>
<td>53°03'47&quot;N 06°06'05&quot;W</td>
<td>2011</td>
<td>Nectar, flowers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>Nectar</td>
</tr>
<tr>
<td>Moate Park</td>
<td>Roscommon</td>
<td>53°35'39&quot;N 08°09'46&quot;W</td>
<td>2011</td>
<td>Nectar, flowers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>Nectar</td>
</tr>
<tr>
<td>Shankhill</td>
<td>Wicklow</td>
<td>53°11'37&quot;N 06°25'36&quot;W</td>
<td>2011</td>
<td>Nectar, flowers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>Nectar</td>
</tr>
<tr>
<td>Trooperstown</td>
<td>Wicklow</td>
<td>53°01'06&quot;N 06°16'27&quot;W</td>
<td>2011</td>
<td>Flowers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>Flowers</td>
</tr>
</tbody>
</table>

Figure C.1. Average sugar concentrations (molarity) of composite *R. ponticum* nectar samples collected over two years from three Irish populations. Sugar concentrations were measured using HPLC (conducted using a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA) and an ED40 Electrochemical detector (Dionex, Sunnyvale, California, USA)).
Table C.2 Oral LC$_{50}$ values (concentration at which 50 percent of individuals experience death at a given time point) of GTX I for honeybees (A. mellifera mellifera). Assays were carried out according to the US Environmental Protection Agency guidelines for the acute toxicity testing of honeybees (USEPA 1996). Individuals were harnessed and dosed with 20 μL of one of six treatments: 0.44 μg/mg GTX, 0.22 μg/mg GTX, 0.11 μg/mg GTX, 0.055 μg/mg GTX, 0.0275 μg/mg GTX and a control sugar solution containing no GTX (n=30 bees per level, divided equally into 5 trials). Data were analyzed using logit regression modelling.

<table>
<thead>
<tr>
<th>Time</th>
<th>LC$_{50}$ (μg/mg)</th>
<th>95% Confidence limits</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hour</td>
<td>0.212</td>
<td>0.154-0.325</td>
<td>153</td>
</tr>
<tr>
<td>48 hour</td>
<td>0.172</td>
<td>0.122-0.269</td>
<td>153</td>
</tr>
</tbody>
</table>
Appendix D: Accompanying Chapter 4

Table D.1. Species codes and long hand for pollinator guilds represented in Figure 4.5.

a. Insects

<table>
<thead>
<tr>
<th>Code</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>Andrena</td>
</tr>
<tr>
<td>X2</td>
<td><em>Apis mellifera</em></td>
</tr>
<tr>
<td>X3</td>
<td>Baccha elongata</td>
</tr>
<tr>
<td>X4</td>
<td>Bombus hortorum</td>
</tr>
<tr>
<td>X5</td>
<td>Bombus jonellus</td>
</tr>
<tr>
<td>X6</td>
<td>Bombus lucorum aggregate</td>
</tr>
<tr>
<td>X7</td>
<td>Bombus pascuorum</td>
</tr>
<tr>
<td>X8</td>
<td>Bombus pratorum</td>
</tr>
<tr>
<td>X9</td>
<td>Episyrophus balteatus</td>
</tr>
<tr>
<td>X10</td>
<td><em>Erioza syrphoides</em></td>
</tr>
<tr>
<td>X11</td>
<td>Eristalis pertinax</td>
</tr>
<tr>
<td>X12</td>
<td>Lasioglossum</td>
</tr>
<tr>
<td>X13</td>
<td>Leucozona lucorum</td>
</tr>
<tr>
<td>X14</td>
<td><em>Melanostoma/Platycheirus</em></td>
</tr>
<tr>
<td>X15</td>
<td>Meliscaeva</td>
</tr>
<tr>
<td>X16</td>
<td>Parasyrphus annulatus</td>
</tr>
<tr>
<td>X17</td>
<td><em>Pieris napi</em></td>
</tr>
<tr>
<td>X18</td>
<td>Rhingia campestris</td>
</tr>
<tr>
<td>X19</td>
<td><em>Sericomyia lappona</em></td>
</tr>
<tr>
<td>X20</td>
<td><em>Sericomyia silentis</em></td>
</tr>
<tr>
<td>X21</td>
<td>Sphegina clunipes</td>
</tr>
<tr>
<td>X22</td>
<td><em>Syrphus</em></td>
</tr>
<tr>
<td>X23</td>
<td>Unidentified moth</td>
</tr>
<tr>
<td>X24</td>
<td>Unidentified syrphid</td>
</tr>
<tr>
<td>X25</td>
<td><em>Volucella pellucens</em></td>
</tr>
<tr>
<td>X26</td>
<td>Xylota</td>
</tr>
</tbody>
</table>

b. Plants

<table>
<thead>
<tr>
<th>Code</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Rhododendron ponticum</em></td>
</tr>
<tr>
<td>2</td>
<td><em>Leycesteria formosa</em></td>
</tr>
<tr>
<td>3</td>
<td>Cardamine pratensis</td>
</tr>
<tr>
<td>4</td>
<td>Cirsium palustre</td>
</tr>
<tr>
<td>5</td>
<td>Conopodium majus</td>
</tr>
<tr>
<td>6</td>
<td><em>Cytisus scoparius ssp. scoparius</em></td>
</tr>
<tr>
<td>7</td>
<td>Digitalis purpurea</td>
</tr>
<tr>
<td>8</td>
<td>Filipendula ulmaria</td>
</tr>
<tr>
<td>9</td>
<td>Galium aparine</td>
</tr>
<tr>
<td>10</td>
<td>Geranium robertianum</td>
</tr>
<tr>
<td>11</td>
<td><em>Glechoma hederacea</em></td>
</tr>
<tr>
<td>Species</td>
<td>Average Abundance R1</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Rhododendron ponticum</td>
<td>22.48</td>
</tr>
<tr>
<td>Stachys sylvatica</td>
<td>0</td>
</tr>
<tr>
<td>Hyacinthoides non-scripta</td>
<td>6.78</td>
</tr>
<tr>
<td>Digitalis purpurea</td>
<td>1.03</td>
</tr>
<tr>
<td>Rubus fruticosus</td>
<td>0</td>
</tr>
<tr>
<td>Veronica chamaedrys</td>
<td>4.64</td>
</tr>
<tr>
<td>Galium aparine</td>
<td>0</td>
</tr>
<tr>
<td>Stellaria graminea</td>
<td>0</td>
</tr>
<tr>
<td>Hypericum androsaemum</td>
<td>0.2</td>
</tr>
<tr>
<td>Lonicera periclymenum</td>
<td>0.22</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>0</td>
</tr>
<tr>
<td>Ranunculus repens</td>
<td>3.78</td>
</tr>
<tr>
<td>Scrophularia nodosa</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table D.2 The contribution of each plant species to the composition of floral resources at sites invaded by *R. ponticum* in round 1 vs. 2 of sampling as determined by SIMPER (Similarity of Percentages) analysis. Data were square root transformed.
Table D.3  The contribution of each insect taxon to communities at sites invaded by *R. ponticum* in round 1 vs. 2 of sampling, as determined by SIMPER (Similarity of Percentages) analysis. Data were square root transformed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean interaction frequency R1</th>
<th>Mean interaction frequency R2</th>
<th>Percent contribution</th>
<th>Cumulative percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Meliscaeva</em></td>
<td>0.46</td>
<td>0.95</td>
<td>12.95</td>
<td>12.95</td>
</tr>
<tr>
<td><em>Bombus lucorum aggregate</em></td>
<td>1.01</td>
<td>0.36</td>
<td>10.91</td>
<td>23.86</td>
</tr>
<tr>
<td><em>Sphegina clunipes</em></td>
<td>1.08</td>
<td>0.41</td>
<td>9.47</td>
<td>33.33</td>
</tr>
<tr>
<td><em>Bombus pratorum</em></td>
<td>0.65</td>
<td>0.11</td>
<td>8.66</td>
<td>41.99</td>
</tr>
<tr>
<td><em>Eristalis pertinax</em></td>
<td>0.37</td>
<td>0.44</td>
<td>6.52</td>
<td>48.51</td>
</tr>
<tr>
<td><em>Episyrphus balteatus</em></td>
<td>0.31</td>
<td>0.35</td>
<td>4.91</td>
<td>53.42</td>
</tr>
<tr>
<td><em>Sericomyia silentis</em></td>
<td>0.19</td>
<td>0.29</td>
<td>4.88</td>
<td>58.3</td>
</tr>
<tr>
<td><em>Syrphus</em></td>
<td>0.33</td>
<td>0.57</td>
<td>4.66</td>
<td>62.96</td>
</tr>
<tr>
<td><em>Eriozona syrphoides</em></td>
<td>0.36</td>
<td>0</td>
<td>4.57</td>
<td>67.53</td>
</tr>
<tr>
<td><em>Xylota</em></td>
<td>0.32</td>
<td>0.13</td>
<td>3.59</td>
<td>71.13</td>
</tr>
</tbody>
</table>
Appendix E: Accompanying Chapter 5

Figure E.1. *R. ponticum*-dominated study sites (black circles) in the native range in Spain (Cádiz province) and the introduced range in Ireland (county Wicklow).
Figure E.2  Percent cover estimates of *R. ponticum* in sites in the a. native (Spanish) and b. introduced (Irish) range, estimated using 20 X 20 m quadrats. Value represent mean percent cover estimate per site, vertical bars show the standard error of each mean.
Figure E.3 Percent of insect individuals (dark grey) and flower visits (light grey) attributed to each guild to all plant species present in communities in a. the native (Spain) and b. introduced (Ireland) range of invasive *R. ponticum*. 
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