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Chemical ecology and conservation biogeography of *Rhododendron ponticum* L.

Thesis submitted for the degree of Doctor of Philosophy (Ph.D.)

2015

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Paul Anthony Egan

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Summary

The general aims of this thesis were two-fold; firstly, to further knowledge on the topic of toxic nectar in plants, utilizing *Rhododendron ponticum* (and associated taxa) as a model for these investigations; and secondly, to assess climatic suitability for this species within its native range under climate change, as a model scenario to inform best-practice in conservation. This shrubby species occurs as a Tertiary relict in the Iberian Peninsula, but is widely naturalised and invasive throughout Ireland, Britain and parts of NW Europe. This contrasting duality in distribution afforded the opportunity for a number of comparative assessments to be made between the native and invasive range of *R. ponticum*; and therefore constituted the basis of a number of research questions that were assessed.

Despite an assumed importance in regulating plant interactions with both floral mutualists and antagonists, there has been little fundamental investigation into how expression of toxic (or deterrent) compounds in nectar may vary in spatial terms. To investigate this topic, variation of two diterpenoid grayanotoxins (GTX I and GTX III) in nectar of *R. ponticum* was examined, within and between the species' native and invasive range. Quantification of GTXs was undertaken through LC-MS analysis, and habitat and climatic data collected in the field and assembled from bioclimatic datasets. Large geographic variation in nectar GTX I, but not GTX III, was revealed between ranges. The native plants exhibited what to-date represent some of the highest known concentrations of toxic compounds in nectar, whereas in contrast, invasive plants exhibited reduced expression or a complete absence of nectar GTX I. While abiotic variables explained some variation in GTX I levels, these could not account for this observed discrepancy. Spatial aggregation of low GTX-producing plants was in addition detected, which may facilitate pollinator foraging and/or the avoidance of exposure to nectar toxins.

In order to understand this large observed variation in expression of nectar GTX I between ranges, a second study was devised which sought to examine natural selection on toxin levels in nectar, as well as on that of flowers and leaves. The potential role of both mutualist and antagonist biotic agents in driving this selection was explored through phenotypic selection analysis, and experimental bioassays with GTX I and pollen supplementation experiments conducted in the field. Selection acting on GTX I levels in nectar, but not other tissues, was found, implicating pollinators, and not herbivores as the main mediators. This study therefore represents the first empirical evidence of direct pollinator-mediated selection on a plant chemical defence.

From these findings on the microevolutionary ecology of toxic nectar, I then progressed to a wider macroevolutionary scale of focus, in a subsequent investigation involving a broad sample of species of *Rhododendron* adapted to various different pollinator types. The main aims of this study were to assess the relative frequency as to which toxic nectar may be encountered amongst plants adapted to different pollinator types, as well as examine patterns of trait evolution in both toxic nectar and pollination syndromes through phylogenetic comparative methods. Results indicated that co-evolution of specialized pollination syndromes has reduced the prevalence of toxic nectar amongst such species, and that species which have adapted to pollination through different pollen vectors (such as bee, bird, butterfly or moth pollination), hold different likelihoods of expressing toxic nectar. Ancestral state reconstruction affirmed these patterns; in that loss of toxic nectar represents an evolutionarily advanced character in plants.

Finally, I examined the potential threats to *R. ponticum* (subspecies *baeticum*) in the species' native Iberian range, where it is a narrow endemic of relict habitat. The species' juxtaposition as an invasive alien species threatened with extinction in its native range could prove useful to demonstrate both the potential risks and benefits associated with translocation as a conservation tool. Simulations of future migration for *R.p.* subsp. *baeticum* under several climate change scenarios were initiated utilizing species distribution models (SDMs) coupled to a population spread model. The main findings suggest the distinct possibility of climate-induced extinction of this taxon through loss of suitable climate in areas of its current occupation, coupled with an inability to migrate into areas of future suitable climate. Options for conservation were then explored, which included identification of species-specific microrefugia in which to potentially implement 'assisted migration'. The framework developed as part of this study was successful in identifying candidate sites potentially suited for conservation translocation by the end of the current climate period (i.e. 2020).

To conclude, a synthesis of results from these two broad investigations on *R. ponticum* (and associated taxa) is presented, highlighting, in particular, their larger relevance to the fields of chemical ecology and conservation biogeography.

To Sonia, Fionn and my family

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List of main contributors

Chapter	Contribution
1	• Chapter written by PE, with comments by JS
2	• Experiments conceived and designed by PE, JS, GW & PS • Data collected by PE • Chemical analysis by PE, PS & FB • Statistical analysis by PE & JS • Chapter written by PE, with comments by JS, GW & PS
3	• Experiments conceived and designed by PE, JS, MG, GW & PS • Data collected by PE & EJT • Chemical analysis by PE & PS • Statistical analysis by PE • Chapter written by PE, with comments by JS, EJT & PS
4	• Experiments conceived and designed by PE, GW, JS, & PS • Data collected by PE, GW & PS • Chemical analysis by PE & PS • Statistical analysis by PE, JS & GW • Chapter written by PE, with comments by JS
5	• Experiments conceived and designed by PE, DB, JS, WT • Data collection & processing by PE, DB, DG, JR • Statistical analysis by PE & DB • Chapter written by PE, with comments by JS, DB & WT
6	• Chapter written by PE, with comments by JS

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Contents

Chapter 1

General Introduction

"Oh, they ate and drank and bragged no end
about what they were gonna do,
while they scarfed up Rhododendron Honey
and Amanita Stew."

– Leslie Fish

1 General Introduction

The role of natural scientists in documenting life on this planet is broad: from elucidation of past patterns of evolution and contemporary microevolutionary forces which shape species (Theis and Lerdau 2003, Conner 2006), to practical management of this immense evolutionary wealth and extrapolation of its future trajectory; in devising new conservation strategies to off-set mounting global change (Guisan and Thuiller 2005, Franklin et al. 2014). Assembling such a broad base of knowledge for individual species or groups of taxa necessitates the combined contribution of many disciplinary fields of investigation. However, while we proclaim to live in an age of interdisciplinary science, its genuine application in practice perhaps remains limited (Berenbaum 2014).

Within the body of work encompassed by this thesis, I sought to gain fundamental and practice-relevant insights on a number of research questions through use of a model plant species (*Rhododendron ponticum* L. (Ericaceae) and associated taxa), drawing simultaneously upon phytochemical, ecological, evolutionary and biogeographical methods of investigation. This introductory chapter serves to introduce the taxa and fields of investigations employed throughout the research chapters (Chapters 2-5), with a particular focus on recent developments and some novel lines of thinking currently within each. My research objectives are then explicitly laid out. A concluding discussion chapter (Chapter 6) brings together the main research outcomes, on the basis of their individual merit, but also seeks to explore knowledge gained through overall synergy of these topics, as a unified interdisciplinary investigation.

1.1 Secondary metabolites and plant-environment interactions

An astonishing array of plant secondary metabolites exists in nature. This vast chemodiversity has naturally prompted the desire of organic chemists, plant physiologists, ecologists and others (including briefly plant systematists (Harborne and Turner 1984) during the vogue of chemotaxonomy which preceded the rise of molecular phylogenetics) to organise these natural products into meaningful categories based on various structural attributes; permitting a better understanding of their biosynthesis, taxonomic distribution and functional role in plants. Thus, based on their biogenesis, secondary metabolites are commonly divided into three major groups (Dewick 2002); nitrogen-containing compounds (such as alkaloids, cyanaogenic glycosides and glucosinolates), flavonoids and phenolics (including phenylpropanoids, anthocyanins, and polyphenolic compounds such as tannins), and terpenoids (which are further discerned by the number of five-carbon isoprene units contained in the skeleton, e.g. mono- (C_{10}) and sesquiterpene (C_{15}) volatile essential oils, diterpenes (C_{20}) etc.). To-date, well over 100,000 unique structures have been determined from plants across these three major groups (Mendelsohn and Balick 1995, Wink 2010), and it is believed that a typical species is probably capable of simultaneously expressing some

5,000-20,000 of these; albeit predominantly at trace levels difficult to detect even with modern highly sensitive instrumentation (Trethewey 2004).

Although once thought of as mere end-products of metabolism, generated by plants in lieu of a functional excretory system analogues to that of animals (Hartmann 2007), our understanding of the importance of plant secondary metabolites has increased drastically over the last half century. Although certainly not all possessing an adaptive role (Berenbaum and Zangerl 1996), secondary metabolites are known to perform a host of physiological and ecological functions within plants (in addition to their immense therapeutic value for humans (Mendelsohn and Balick 1995, Egan and van der Kooy 2013)), and play a particularly important role in interactions between plants and their biotic and abiotic environment (Theis and Lerdau 2003). From an ecological viewpoint, the most important of these involve signalling and defensive functions in interactions with herbivore and pathogenic antagonists, and in attracting plant mutualists such as pollinators, seed dispersers, microbial symbionts, and the natural enemies of herbivore attackers within multitrophic interactions (Heil 2008, Harvey et al. 2011). Secondary metabolites also perform many important physiological functions within plants, including hormonal regulation and in protection of plant tissues and cells in adaptive responses to abiotic stress (Ramakrishna and Ravishankar 2011).

1.1.1 Natural variation

The reasons for phenotypic variation in secondary metabolite expression in plants can be broadly divided between those that are functional responses to abiotic and biotic factors, non-adaptive consequences of these factors, or the varying genotypic capacity of plants to express such compounds. Given the significant metabolic costs associated with the biosynthesis, transport and storage of many phytochemicals (Gershenzon 1994), and that herbivory is often highly variable in space and time (Schoonhoven et al. 2005); plants have accordingly evolved mechanisms to minimize the unnecessary production of costly chemical defences. Hence, these may be either 'constitutive' (which are maintained at relatively stable levels of expression), or 'inducible' (which are either rapidly produced upon a plants sense of attack, or steadily built up thereafter). Constitutive and induced compounds can function in different roles against generalist and specialist herbivores, which possess diverse behavioural strategies and physiological mechanisms to surmount toxic or deterrent antifeedant compounds (Nishida 2002, Després et al. 2007). Although they may form an effective defence against generalists, certain phytochemicals can also be co-opted by specialists as cues for locating host plants for ovipositioning or feeding (Smallegange et al. 2007, Talsma et al. 2008). Dynamic scenarios such as this demonstrate how large variation in secondary metabolites may be maintained in plant populations – as selection should favour increased defence concentration in the presence of generalists but not specialists; whereas in the presence of both herbivore guilds, selection should favour an intermediate concentration (Lankau 2007). As no permanent pattern of selection may be in action, the genes for defence traits can hence remain variable over time. While 'resistance' to herbivory through expression of secondary metabolites forms a major defensive strategy, 'tolerance' of attack can also feature as a complementary defence co-employed by plants. Indeed 'mixed' defensive strategies in certain circumstances may afford the most optimal form of defence selected for in plants (Carmona and Fornoni 2013).

Variation in secondary metabolite production may also reflect differences in a plant's abiotic environment and/or ability to acquire nutrients or intercept light e.g. due to canopy cover or topographic features such as aspect (Cates 1996). Many phytochemicals (such as anthocyanins, polyphenolic antioxidants etc.) also function as plastic traits expressed in response to abiotic stimuli (including drought, UV irradiation and other stressors) permitting adaptation to these specific environmental conditions (Gershenzon 1984). Several theories put forward over the last decades have sought to examine if and why plants up-regulate production of certain classes of secondary metabolites under certain abiotic conditions (i.e. the carbon-nutrient balance hypothesis), and how these are allocated differently across different tissues types for defensive purposes (i.e. optimal defence theory). Support for the carbon-nutrient balance hypothesis has in recent years waned, as its general failure to predict relative expression of defensive compound types, as a function of resource availability, has become clearer (Hamilton et al. 2001, Koricheva 2002). In contrast, a recent meta-analysis has upheld the validity of the optimal defence theory (McCall and Fordyce 2010), that regardless of the influence of several factors (such as growing conditions, position on plant etc.), tissues which are more valuable to plants (in terms of influencing fitness) exhibit consistently higher relative levels of chemical defence.

Despite detailed knowledge of their functional importance in plants, however, the question still remains as to why expression of many secondary metabolites varies so extensively, including spatially (between taxa, habitats and populations, down to plant individual and organ levels) and temporally (over individual lifetimes, within and between phenological seasons, and across ontogenetic phases of tissue development). While such topics have provided fruitful avenues of investigation in chemical ecology over the last decades (Wink 2010), there nonetheless exists the potential for many new discoveries. Untangling the causes and consequences of variation at the intraspecific level, in particular, remains a key challenge (Moore et al. 2014).

1.1.2 Antagonists, mutualists and floral chemistry

While traditionally the majority of focus on chemical defence has concerned herbivory of plant vegetative structures, an increasing awareness has more recently emerged of the defensive needs of flowers, and the negative fitness impacts incurred from florivory (Strauss 1997, McCall and Irwin

2006). These negative impacts on fitness can arise either directly, as a result of consumption of floral structures which impact upon reproduction, or indirectly, through influencing the attractiveness of flowers to pollinators, which can be deterred by even small levels of damage (Mothershead and Marquis 2000). The latter indirect effects are hence likely to be more pronounced in obligatory or predominantly out-crossing plant species, which due to a heavier reliance on entomophilous pollination may experience pollen limitation of seed production (Knight et al. 2005). Plants must therefore chemically defend floral structures. In concordance with optimal defence theory, flowers may frequently also possess the highest levels of expressed defence, relative to other tissue types (Spitaler et al. 2006, Egan and van der Kooy 2012).

Floral nectar and, to a lesser extent, pollen function as rewards designed to attract mutualists as pollen vectors, in promotion of sexual reproduction in plants via out-crossing. While the presence of secondary metabolites in nectar has been inferred for centuries, due to outbreaks of 'toxic' honey poisoning (Ott 1998, Koca and Koca 2007), this subject has only recently received serious attention in ecological and evolutionary terms within the last two decades (Adler 2000). Several diverse explanations have been offered as to why so-called toxic nectar is produced by plants (Rhoades and Bergdahl 1981, Adler 2000), including a mere passive leakage from phloem sap (Lohaus and Schwerdtfeger 2014). However, most explanations pose an adaptive significance to this phenomenon. Ascribed ecological functions include (on a non-mutually exclusive basis): defence against nectar robbing (Stephenson 1981); promotion of pollination fidelity by specialist pollinators (Masters 1991); reduction of self-pollen transfer (Irwin and Adler 2008, Kessler et al. 2012); increased pollinator visitation due to an advantageous physiological action conferred by 'toxins' (Manson et al. 2010, Wright et al. 2013); in permitting a shift in energy investment from sugar to less costly attractants (Liu et al. 2007); and as antimicrobial defence against bacteria and nectar-dwelling yeasts (Sasu et al. 2010). In contrast to these ascribed beneficial roles, toxic nectar may also prove maladaptive in cases where its ecological costs outweigh such benefits in plants, for example where legitimate pollinators are deterred at the expense of plant reproductive fitness (Adler and Irwin 2005, 2012). This deterrence may depend on ecological context, however, such as the availability of alterative nectar resources to pollinators (Gegear et al. 2007). It is conceivable therefore that some pollinators may only tolerate toxic nectar given scarce foraging opportunities, but avoid it otherwise.

While several adaptive functions are ascribed to toxic nectar, as listed; evidence nonetheless supports the notion that expression of secondary metabolites in nectar is to varying degrees confounded by phenotypic correlation with other plant tissues, such as with leaves and flowers (Irwin and Adler 2006, Manson et al. 2012). The ecological and evolutionary ramifications of this evident non-independent expression of toxins in nectar may potentially be large. For instance, where leaves come under herbivore attack, nectar may also experience systemic expression of induced anti-herbivore defences, to negative consequences (Adler et al. 2006, Halpern et al. 2010). Due to this apparently large evolutionary constraint between expression of secondary metabolites in leaves and nectar, this topic thus provides an ideal opportunity to investigate what may constitute opposing selection pressures on plant chemical defence from antagonist and mutualist sources. To-date, despite its large ecological importance, no investigation has sought to examine natural selection on toxic nectar. Studies of this nature may hold wider implications generally; as floral chemistry remains "perhaps the most underappreciated aspect of pollination biology" (Agrawal 2014).

1.2 Conservation biogeography

Conservation biogeography is defined as the application of biogeography in conservation (Ladle and Whittaker 2011); and in broad terms encompasses the study of the distribution of biodiversity, at landscape scale and above, applied in particular to issues of conservation. The emergence of these topics of investigation in recent decades has been greatly aided by the development of tools and techniques such as Geographic Information Systems (GIS) and species distribution modelling (SDM), which have permitted ever more complex and computationally intensive analyses over large scales and at high resolution. These developments have resulted in new abilities to tackle ever more complicated questions in relation to biodiversity conservation, and as a result, to arrive at solutions which would not have been possible to achieve before. For instance, the practical utility of SDMs in conservation biology is extremely diverse (Guisan and Thuiller 2005), and commonly features in tasks such as the design of ecological corridors or setting protected area boundaries, predicting the likelihood of where new populations of rare or endangered species may best be located, in assessment of invasive species and their potential spread, and quantifying changes in habitat suitability for target taxa under climate change. The above listed tasks may be undertaken as part of purely fundamental tests of theoretical hypotheses (Austin 2007), ranging all the way to fully applied applications, to inform practical decision-making processes and conservation planning (Franklin et al. 2014).

1.2.1 Climate change and assisted migration

A central paradigm of mainstream conservation is the aim to preserve biota within their current distributional range. A major challenge to this goal is presented in the form of climate change. The large shifts in global climate potentially witnessed this century could drastically undermine conservation efforts, and therefore present a whole new set of challenges which must be addressed. For example, how effective will national parks be at preserving protected species in the near future under climate change, and what may be the fate of species such as narrow endemic alpines and other climate specialists? As suitable habitat for most species in the northern hemisphere is generally anticipated to shift northwards under what (for the most part) will be relatively hotter and drier conditions (IPCC 2013, Kirtman et al. 2013), the extent to which species are capable of adequately tracking these climate paths northwards via natural migration is for many species questionable (Early and Sax 2011, Mahlstein et al. 2013), and for others very unlikely (Reinhardt et al. 2005).

Where such projections of habitat suitability under future climate change are conducted for species, and resultant outcomes are far from positive, the question is therefore presented as to what response, if any, is justified in order to off-set climate-mediated extirpations. Given the magnitude to which climate change is likely to act in this century, this scenario is therefore unprecedented in conservation terms, and represents a real and urgent challenge. Hence, in recent decades there has in response been increasing calls for new strategies which are equally unprecedented in their nature – and some of which challenge the traditional paradigm of conservation (Griffith et al. 1989, Hunter 2007).

One such emergent conservation strategy is 'assisted migration' of climate-threatened species. This strategy encompasses the human-mediated movement of species into new 'climate-appropriate' regions, which may exist within or beyond areas of a species' historical range. Although the validly of this strategy remains strongly contested at present on both sides of the debate (Jørgensen 2011, Dalrymple and Moehrenschlager 2013), it has nonetheless begun to see practical implication in the wild (Marris 2008). Those opposed to translocation generally argue from the point of view that such interventions are 'unnatural', and that the benefits of intentionally releasing species into new geographic areas do not outweigh the risks (such as the potential for subsequent invasion) (Ricciardi and Simberloff 2009b). On the other hand, proponents see this technique as a last line of action necessary to avert widespread climate-induced extinctions, and hold that within the perspective of geological time, and in context of climate change; "there is nothing 'special' about conserving species within their current distributions" (Thomas 2011). As a whole, translocation science still remains in its infancy, as a coherent conservation strategy, and there is much to-do in building on established guidelines and setting best practice.

1.3 *Rhododendron* and the family Ericaceae

The Ericaceae (or heath) family contains about 120 genera comprising over 4,000 species (Kubitzki 2004). The family exhibits an almost cosmopolitan distribution worldwide throughout sub-tropical and tropical regions at high elevation, and temperate areas of both northern and southern hemispheres (Kron and Chase 1993). The largest member of the family, *Rhododendron* L., contains some 1,025 species, and together with the speciose genera *Erica* L. (ca. 860 species) and *Vaccinium* L. (ca. 450 species) constitute over half of total species. The family contains several well known horticultural and economically important genera (Wiersema and Leon 1999). These include *Vaccinium* spp. valued as fruit crops, such as cranberries (of subgenus *Oxycoccos*), and blueberries (subgenus *Vaccinium*). Also of economic importance is the genus *Gaultheria* (wintergreen) which is grown commercially for its aromatic essential oil, and genera with various species cultivated for ornamental purposes, such as *Rhododendron* (syn. Azalea), *Pieris* (Andromeda), *Kalmia* (Mountain Laurel), *Empetrum* (Crowberry), and *Erica/Calluna* (Heather).

Rhododendrons may be either evergreen or seasonally deciduous, and several diverse growth-forms are found, including dwarf and woody shrubs, small to large trees, and epiphytes. The vast majority of species thrive in acidic, nutrient-impoverished soils in well known symbiotic association with specialised Ericoid mycorrhiza (Read 1983, Straker 1996). This mycorrhizal association is considered crucial or even obligatory for the success of plants which benefit from transfer of mineral nutrients. This symbiosis has allowed growth and survival in a variety of otherwise stressful abiotic or competitive habitats (Cairney and Meharg 2003) – which range from heath and tundra, to sub-alpine scrub, as well as open temperate or tropical forests.

Common morphological traits within the genus include spirally arranged leaves with entire margins, and large hermaphrodite flowers adapted to entomophilous pollination (Mejías et al. 2002, Escaravage and Wagner 2004, Stout et al. 2006, Ono et al. 2008, Kudo et al. 2011). While most species thus show an adaptation to bee-pollination (melittophily); species of *Rhododendon* section Vireya (colloquially known as tropical rhododendrons) have adapted to a much wider range of specialized pollen vectors, and exhibit floral adaptations to butterflies (psychophilous pollination – i.e. scented, brightly coloured corollas, with a landing pad and long tube corresponding to insect tongue length), sphingid moths (phalaenophilous pollination – i.e. usually heavily scented white, and nocturnally flowering, with tubular corollas) and birds (ornithophilous pollination i.e. with red or orange-red corollas which frequently exhibit curvature suited to probing by beaks). However, these pollination syndromes have mostly been assumed based on floral morphology only, as observational studies of pollination ecology in Vireya have been scarce (Stevens 1976, Stevens 1985) – in contrast to those of bee-pollinated species in the genus.

1.3.1 Chemical defence and grayanotoxins

While a diverse array of secondary metabolites are known to accumulate in most (if not all) plant parts of *Rhododendron*; diterpenoids are generally the most important (in both ecological and pharmacological terms) and abundant of phytochemicals (Qiang et al. 2011, Li et al. 2013). High concentrations of tannins and other polyphenolics are also a well known feature of the genus, which may act as antinutritional deterrents against insects, or (together with soil lipids) as allelopathic growth suppressants against neighbouring plant competitors (Rotherham 1983, Nilsen et al. 1999, González-Pérez et al. 2011, Davis 2013). The bioactivity of terpenoid constituents of *Rhododendron* has been well studied (Popescu and Kopp 2013), and the renowned pharmacological action and potent toxicity of many species is predominantly ascribed to the presence of diterpenes and triterpenoids – although large amounts of monoterpene volatile oils localised in glandular trichomes and leaf scales may also play a biologically relevant role in plant defence against herbivores and pathogens (Doss 1987, Usta et al. 2012). Plant terpenoid compounds are produced and subsequently modified from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) precursors (Fig. 1); fundamental five-carbon isoprene units derived from the mevalonate and deoxyxylulose phosphate pathways.

Grayanotoxins (GTXs) are a particular class of *ent*-kaurane diterpenoid, which are polyhydroxylated hydrocarbons with a rigid tetracyclic skeleton found with or without glycosides (formally known as andromedotoxins, acetylandromedols or rhodotoxins). The exact number of these compounds isolated to date is not known but is estimated to be in the order of 70, which in spite of constant new discoveries still appear restricted to the Ericaceae (Jawad et al. 1977, Harborne and Baxter 1993, 1996, Li et al. 2013). However, tetracyclic diterpenes with a similar skeleton have recently been found in the related family Eurphorbiaceae (Ferreira et al. 1998), and it is currently unclear whether this feature represents a common biogenesis due to shared ancestry or chemical convergence.

The advanced stages of the biosynthetic pathway of grayanotoxins remains incompletely known, but originates initially from condensation of DMAPP and IPP in plant chloroplasts to form geranylgeranyl pyrophosphate (GGPP), the general precursor of numerous diterpene families (Fig. 1). Rearrangment and modification of the *ent*-kaurene skeleton produces a diverse range of tetracyclic diterpene types, which in addition to grayanotoxins include the gibberellins, universal in plants as growth hormones. In addition to GTXs, three other structurally related *ent*-kaurane-type diterpenes are mainly found in the Ericaceae, namely leucothanes (6/6/6/5 ring system), kalmanes (5/8/5/5 ring system) and various 1,5-secograyanane derivatives (10/6/5 ring system).

GTXs have in many cases been found as the agents responsible for the extreme toxicity exhibited by a number of Ericaceae genera, and are usually present in abundance in most species of *Rhododendron*. Although the toxicity of many Ericaceous species has long been known, the modern study of grayanotoxins only stretches back to the 1950s, when grayanotoxin I (GTX I) was first definitely isolated (Moran et al. 1954, Wood et al. 1954, Tallent et al. 1957). Affirmation as to the precise structure of GTX I and GTX III (Kakisawa et al. 1961) was instrumental in the subsequent

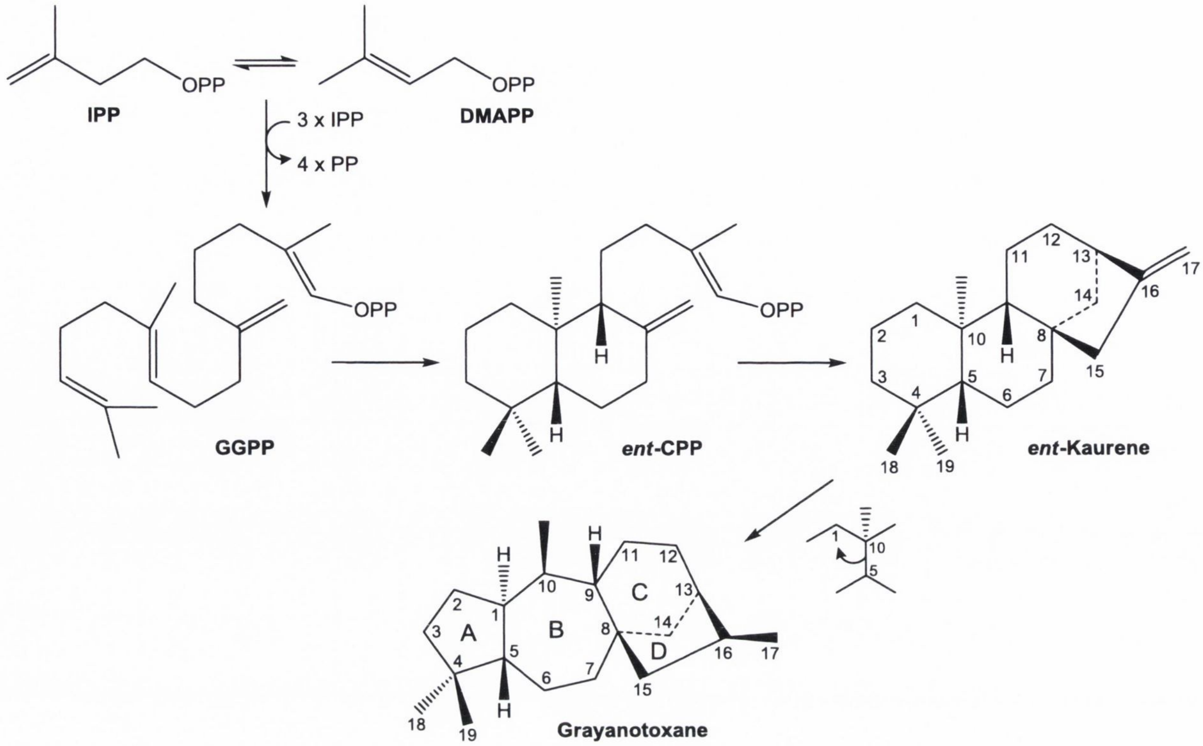

Figure 1. Biosynthetic pathway of grayanotoxins from 5-carbon isoprene precursors. GGPP undergoes subsequent ring cyclization and via the bicyclic intermediate *ent*-copalyl pyrophosphate (*ent*-CPP) leads to formation of the tetracyclic product *ent*-kaurene. The possible intermediate steps preceding formation of grayanotoxins remain unclear. It is assumed however that bond migration in the *ent*-kaurene skeleton between the C-5/C-10 and C-1 carbon ultimately leads to the formation of grayanotoxins (Masutani et al. 1981), with a 5/7/6/5 ring system exhibiting a characteristically enlarged B ring.

escalation in discovery of new grayanotoxins which ensued (Kurten et al. 1970, Gasa et al. 1972, Jawad et al. 1977). Leading on from this was conclusive evidence of the general widespread occurrence of GTXs in many Ericaceae known to be poisonous to humans and animals (Constantine et al. 1967, Loriaux et al. 1973, Gasa et al. 1976).

GTXs are known to exhibit both toxic and deterrent effects against a broad range of phytophagous taxa (including model Lepidoptera and Coleoptera such as fall armyworm (*Spodoptera frugiperda* J.E. Smith), cabbage white butterfly *(Pieris rapae* L.), gypsy moth (*Lymantria dispar* L.), Colorado potato beetle (*Leptinotarsa decemlineata* Say) and others (Elnaggar et al. 1980, Klocke et al. 1991, Hu et al. 1993, Zhang et al. 2005, Zhong et al. 2006)) which in parts of China has justified their use as a natural insecticides derived from *Rhododendron* flowers. GTXs act as neurotoxins in a wide range of organisms (from invertebrates to vertebrates and mammals) due to immobilization of sodium channel receptors: GTXs are known to bind to voltage gated sodium channels in their open state (Takeda and Narahashi 1988, Gunduz et al. 2008). It has been noted, however, that the efficacy of bioactivity may be due to only a few important functional groups in the molecule, as even small structural modifications have been observed to hold a large impact on the insecticidal activity of

GTXs (Hu et al. 2000). Investigations to-date focused on the ecological function of grayanotoxins have reported that a number of specialist geometrid moths have evolved the capacity to detoxify GTXs through sequestration, which is also known to enhance the unpalatability (and hence resistance) of these moths against predators (Nishida 2002). Elsewhere, observational studies of natural herbivores of *Rhododendron ponticum* have indicated that this species usually exhibits little sign of herbivory, but that a small number of generalist weevil species may be responsible for at least some of this damage (Judd and Rotherham 1992, Yela and Lawton 1997, Willott and Compton 1998). Despite their wide distribution and potential ecological importance amongst many Ericaceous species, however, relatively little remains known on the chemical ecology of GTXs, and further studies are thus required in this regard.

1.3.2 Rhododendron ponticum as a model system

Rhododendron ponticum was used as a study species (albeit a wider focus on the genus as a whole is presented in Chapter 5). This shrubby species occurs as a Tertiary relict in the Iberian Peninsula (as *R.p.* subsp. *baeticum* (Boiss. & Reut.) Hand.-Mazz.); where it is constrained to highly specific refugia habitat within a disjunct range featuring the Aljibe Mountains, north of the Strait of Gibraltar in Spain, and the Monchique and Caramulo Mountains of south and mid Portugal respectively (Castsroviejo 1997). The narrow endemic distribution exhibited by this species has thus justified its designation on national Red Lists in both Spain and Portugal, as endangered and vulnerable respectively (Walter and Gillett 1998, Blanca et al. 1999).

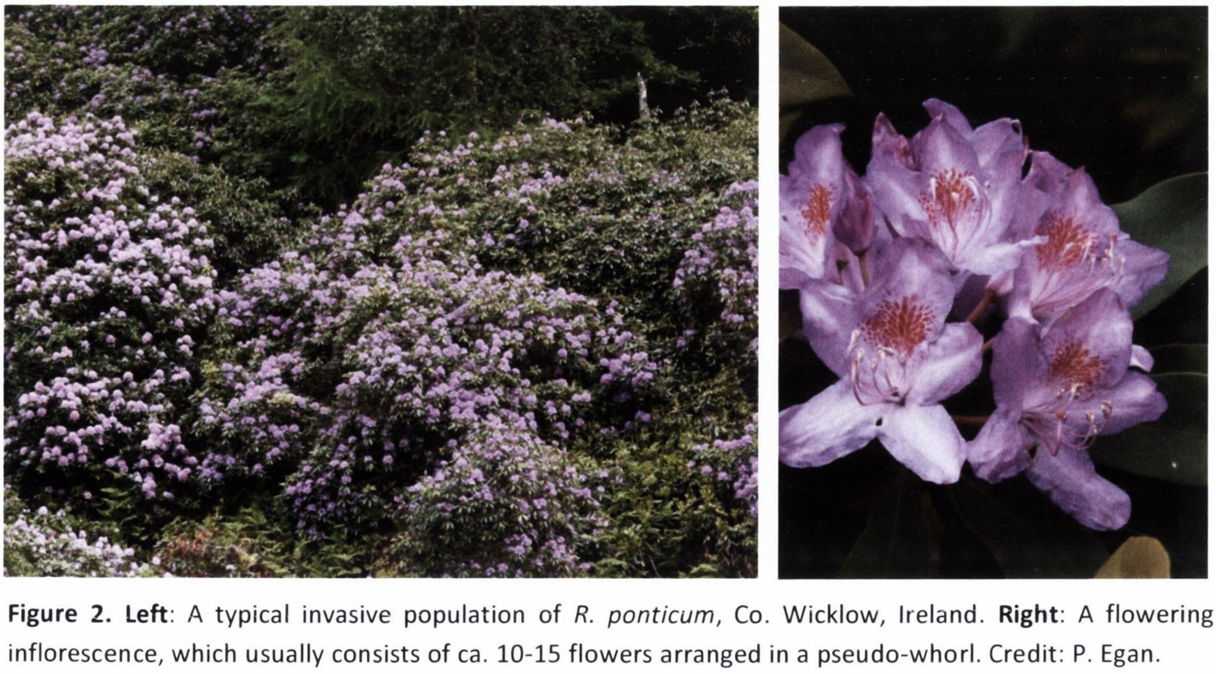

Figure 2. Left: A typical invasive population of *R. ponticum*, Co. Wicklow, Ireland. **Right**: A flowering inflorescence, which usually consists of ca. 10-15 flowers arranged in a pseudo-whorl. Credit: P. Egan.

In contrast, *R. ponticum* is widely naturalised and invasive throughout Ireland, Britain and parts of NW Europe, from original introductions in the 18th century (Dehnen-Schmutz and Williamson 2006). Although *R. ponticum* also occurs within the Black Sea region (stretching from Bulgaria, through Turkey, to Georgia), molecular analyses have indicated that invasive populations descend from Spanish ancestors (Milne and Abbott 2000), and that these in fact retain both genetic and morphological diversity comparable to that found in the native range (Erfmeier and Bruelheide 2011, Stout et al. 2015). While hybridization between *R. ponticum* and a small number of congeneric species (mainly *R. catawbiense* Michx. and *R. maximum* L.) has been detected in the UK (Milne and Abbott 2000), this process has not been detected in Irish populations (Erfmeier et al. 2011, Stout et al. 2015). In recent times considerable effort has been directed towards its control. This remains severely limited, however, due to both the high cost-factors associated with intervention measures (Dehnen-Schmutz et al. 2004), and the general inaccessibility of many invaded sites (Wong et al. 2002).

This contrasting duality in distribution therefore afforded the opportunity for a number of comparative assessments to be made between the native and invasive range of *R. ponticum*; which constituted the basis of a number of research questions that were assessed. In particular, this species formed a good candidate for assessment of toxic nectar, as the mating system and pollination of *R. ponticum* has been well documented throughout both ranges (Mejías et al. 2002, Stout et al. 2006, Stout 2007b). Plants flower profusely from early summer for a period of ca. 6 weeks, with large pink-purple flowers exhibiting incomplete sequential hermaphroditism (protandry). Although individual plants are self-compatible, a variety of mostly bee pollinators facilitate out-crossing between individuals, resulting in higher relative rates of seed-set (Mejías et al. 2002, Stout et al. 2006). Other factors which support the suitability of this species for studies of toxic nectar include A.) the simplicity of the system; in which toxicity is ascribed to high concentration of a single major (GTX I) and minor (GTX III) compound in nectar (as documented in Chapter 2); B.) a high volume of nectar production – permitting minimal pooling and use of individual plants as the unit of replication, which has not always been possible in such studies of toxic nectar elsewhere (Manson et al. 2012); and C.) a large plant size – permitting a negligible influence of sample collection on the study system; which may have influenced smaller herbaceous species.

1.4 Research objectives

The aims of my research, herein documented as part of this thesis, were two-fold. Firstly, a main focus is given on the topic of toxic nectar (Chapters 2-4), in which I seek to gain a greater understanding of this biological phenomenon, utilising *R. ponticum* as a model system (in addition to a wider set of congeneric species in Chapter 4). Secondly, I further utilise the unique juxtaposition of

R. ponticum (as both an endangered endemic and invasive alien species) in a case-study aimed at assessing current best-practice in conservation translocation (Chapter 5).

Little is known on the extent to which the expression of toxic nectar may vary (in a qualitative and quantitative sense) throughout the geographic range of species, and how abiotic processes may affect this. I examine this fundamental topic in Chapter 2, in asking whether or not observed patterns fit with the generally held adaptive function of toxic nectar. The outcome of this investigation then provides a foundation on which additional questions are posed in subsequent chapters.

Despite its presumably large importance in ecological terms, natural selection either for or against toxic nectar has not been demonstrated. Given that for many species that toxin levels expressed in nectar are not independent of those in leaves; there therefore exists the potential for conflicting selection pressures in plants from mutualists and antagonists. In Chapter 3, I investigate natural selection on toxin levels in both nectar and leaves of *R. ponticum*, and examine whether selection in plants appears overall to be mediated by pollinators via nectar, or herbivores via leaves.

Most studies of toxic nectar to-date have predominantly focused on bee-pollinated species. In comparison, relatively little attention has been afforded to other variously pollinated species. In Chapter 4, I employ a wide sampling of species of *Rhododendron*, focusing on taxa which exhibit diverse and contrasting pollination syndrome types (such as bird, butterfly and moth pollination), and investigate whether the relative frequency as to which toxic nectar is encountered in species is dependent on the pollinator types they are adapted to.

Chapter 5 focuses on the topic of climate change impacts on *R. ponticum* in its native Iberian range. I assess the natural capacity of this species to successfully migrate into future suitable habitat. I demonstrate use of a novel framework developed as part of this case study, which can be more widely employed for purpose of practical identification of species climate change refugia, and in decision-making for conservation translocation.

In a general discussion chapter (Chapter 6) I then seek to synthesize the outcomes of these investigations, in a unified manner, and recommend directions for future research.

Chapter 2

Spatial and environmental correlates of variation in nectar toxin expression

"Even bees, the little almsmen of spring bowers,
know there is richest juice in poison-flowers."

– John Keats

2 Spatial and environmental correlates of variation in nectar toxin expression

2.1 Abstract

Despite a potentially important role in regulating plant interactions, there has been little fundamental investigation on geographic variation of so-called toxic nectar within species. Variation of two diterpenoid grayanotoxins (GTX I and GTX III) in nectar of the invasive *Rhododendron ponticum* (Ericaceae) was here examined, within and between the species' native and invasive range. Large geographic variation in nectar GTX I, but not GTX III, was revealed between ranges; with native plants exhibiting what to-date represent some of the highest known concentrations of toxic compounds in nectar. In contrast, invasive plants exhibited either reduced expression or (in 22% of cases) a complete absence of nectar GTX I. Four environmental variables (irradiance, elevation, aspect and canopy cover) were associated with variation in nectar GTX I, but alone could not account for this geographic discrepancy. Adaptive divergence due to enemy-release from antagonists (including nectar robbers) and/or pollinator-mediated selection offer conceivable hypotheses as to why invasive plants show reduced nectar toxin levels. Spatial aggregation of low GTX-producing plants within populations was also detected, which could facilitate pollinator foraging and/or the avoidance of toxic nectar. Intraspecific variation may represent a common feature amongst toxic-nectar producing species, and the complexity of this variation should be duly acknowledged in future studies which seek to assign bioactivity or adaptive ecological functions to plant chemicals in nectar.

2.2 Introduction

Plants produce toxic or deterrent compounds as a defence against herbivory, and these compounds have been found in most tissue types (McKey 1979, Rasmann et al. 2009, Moreira et al. 2013). Nectar is produced by plants to attract and reward pollinating animals, but can also contain these toxic defence compounds (Baker 1977, Adler 2000). Toxic nectar can, in some cases, deter legitimate pollinators and reduce reproductive fitness (Adler and Irwin 2005, 2012), but can also defend against nectar robbing (Stephenson 1981), promote greater pollinator fidelity and quality/quantity of pollen transfer (Masters 1991, Irwin and Adler 2008, Manson et al. 2010, Kessler et al. 2012, Wright et al. 2013), and act as an antimicrobial defence against bacteria and nectar-dwelling yeasts (Sasu et al. 2010). Many types of toxic secondary metabolites have been reported from nectar and include iridoid and cardiac glycosides (Stephenson 1982, Manson et al. 2012, Lohaus and Schwerdtfeger 2014), toxic or otherwise deterrent phenolics (Baker 1977, Hagler and Buchmann 1993, London-Shafir et al. 2003), a variety of alkaloids of indole, pyrrolizidine, tropane, purine and quinolizidine class (Masters 1991, Kretschmar and Baumann 1999, Adler et al. 2001, 2006, Boros et al. 2010), and structural combinations such as diterpene alkaloids (Gosselin et al. 2013, Manson et al. 2013).

Spatial variation in nectar traits influences the quality and distribution of floral resources encountered by flower visitors (Brenes-Arguedas and Coley 2005), and may affect their behaviour and fitness, as well as the reproductive success of the plant. Local ecological (biotic and abiotic) conditions can affect both the level of toxin expressed in nectar and its effects on flower visitors (Gegear et al. 2007). For example, the concentration of toxic alkaloids in the nectar of domestic tobacco (*Nicotiana tabacum* L.) can vary according to nutrient availability and incidence of leaf herbivory (Adler et al. 2006). Variation among populations in nicotine expression in the nectar of wild tobacco (*Nicotiana attenuata* Torr. ex S.Watson) has also been reported, and plants that express high nicotine levels repel hummingbird pollinators (Kessler et al. 2012). However, knowledge of what constitutes typical intraspecific variation in toxin levels in nectar remains poor, in part to low replication and the restricted geographic scope of most studies.

We investigated variation in nectar toxin expression in *Rhododendron ponticum* L. (Ericaceae), a widely distributed shrub which occurs as a Tertiary relict in the Iberian Peninsula (in disjunct regions of northern and southern Portugal, and southern Spain) and as a naturalised invasive species in NW Europe, following deliberate introductions in the late 1800s (Dehnen-Schmutz and Williamson 2006). As in other Ericaceous genera, rhododendrons produce toxic diterpene secondary metabolites known as grayanotoxins (GTXs – reviewed in Qiang et al. 2011) which are characterised by a polyhydroxylated tetracyclic structure (Masutani et al. 1981). These GTXs have a neurotoxic mode of action, immobilizing sodium channel receptors and preventing inactivation (Takeda and Narahashi 1988, Gunduz et al. 2008). Thus they are toxic to a broad range of vertebrate and invertebrate organisms (Klocke et al. 1991, Puschner et al. 2001) and the toxicity of honey produced from the flowers of *R. ponticum* (known as 'mad honey') has been known since antiquity (Koca and Koca 2007). Flowers of this species produce large volumes of nectar, both in the native and invasive range (Mejías et al. 2002, Stout et al. 2006). The main pollinators of *R. ponticum*, bumblebees (and in particular *Bombus terrestris* L.) (Stout 2007a), are not deterred by levels of GTXs that may be encountered in nectar (Tiedeken et al. 2014), which may account for the invasive success of this species in spite its reliance on animal-pollinated seed production (Stout 2007b).

Naturalised *R. ponticum* populations descend from Spanish ancestors (*R. ponticum* subsp. *baeticum*, Milne and Abbott 2000), and some introgression has occurred between *R. ponticum* and congenerics in Britain, but probably not in Ireland (Erfmeier et al. 2011). Similar to other morphological traits, it is possible that introgression and/or post-introduction evolution has influenced the expression of GTX by *R. ponticum* in its invasive range, but little is known of GTX concentration (or the relative amount of different types of GTX) in the nectar of *R. ponticum* and other rhododendrons (Tallent et al. 1957, Tiedeken et al. 2014). In this study, we tested the following hypotheses that: 1.) there is geographic variation in quantitative and qualitative expression of GTXs in nectar; 2.) plants and populations which are spatially aggregated tend to have similar GTX concentration; and 3.) abiotic conditions in which the plants grow correlate with GTX concentration. Few studies have sought to compare nectar characteristics across the native and non-native range of introduced species (Stout et al. 2006, Ollerton et al. 2012), let alone nectar secondary metabolite content, and this study represents the first such investigation.

2.3 Methods

2.3.1 Sampling locations and data collection

Nectar of *R. ponticum* was sampled from 7-10 plants in each of nine populations in the species' native range (in southern Spain and southern and northern Portugal) and from seven populations of the invasive range in Ireland (Appendix 1). A minimum distance of 20 m was kept between sampled plants in order to avoid sampling clones which can establish through vegetative spread. Collection was made in the field using 1 µl glass microcapillary tubes (Hirschmann, Germany) inserted into the nectar tube at the back of the flower. An average of 1 µl (± 0.7 µl) was extracted per flower, which was pooled to obtain a minimum of 10 µl of nectar from 6-14 flowers (randomly selected to avoid positional bias) per plant. As the level of GTX I in nectar differed according to the floral phase, we standardized sampling to include only mature flowers, i.e. β-phase (Appendix 2). Samples were frozen within 7 h of collection until time of analysis. Care was taken to avoid damage to the flower corolla and nectaries so as not to contaminate samples. In addition, a total of 16 abiotic variables were quantified for every sampled plant at microhabitat or landscape scale, either in the field or derived from bioclimatic datasets (Table 1). To examine temporal constancy in expression, nectar toxin levels were examined between flowering seasons of *R. ponticum*. For this, composite samples of nectar were taken (as described above) from seven plants in one selected population (pop. I2; Appendix 1) in early May 2011; and again one year later in early May 2012.

Table 1. Environmental variables quantified per plant, and their respective resolution and source

Variable & Units	Resolution	Quantification	Reference/Source
Microhabitat scale			
1. Canopy cover (%)	plant level	Measured in the field for each plant through use of a canopy scope	(Brown et al. 2000)
2. Slope (^{O})	30m	Derived from the ASTER Global Digital Elevation Model (GDEM) ver. 2	METI, NASA
3. Aspect – radiation (^{O})	30m	Aspect derived from DEM, and folded to rescale 0-360^{O} to 0-180^{O}, where 180^{O} equates to south, at max irradiance	(McCune and Keon 2002)
4. Aspect – heat load (^{O})	30m	As for 3. – except rescaled to equate 180^{O} to south west, at max heat load	(McCune and Keon 2002)
Landscape scale			
5. Elevation (m)	30m	Derived from the ASTER Global Digital Elevation Model (GDEM) ver. 2	METI, NASA
6. Irradiance (WH/m^{2})	30m	Mean annual irradiance calculated from monthly intervals, accounting for topographic shading	ArcGIS 10.1 Solar Radiation Toolset
7. Tree cover (%)	250m	MODIS Vegetation Continuous Fields – percent tree cover per area	(DiMiceli et al. 2011)
8. Habitat type	300m	Categorized according to the Global Land Cover Map, GlobCover 2009	(Bontemps et al. 2011)
9-12. Precipitation (mm)	1000m	Annual mean and range; max of wettest & min of driest months	(Hijmans et al. 2005)
13-16. Temperature (^{O}C)	1000m	Annual mean and range; max of hottest & min of coldest months	(Hijmans et al. 2005)

2.3.2 LC-MS analysis

Nectar samples were freeze-dried in inserts in vials, and water content recorded as the difference in vial weight (before and after freeze-drying). Dried nectar was then re-suspended in 200 µl MeOH for analysis. LC-MS analysis was carried out on a Waters Alliance LC and ZQ MS detector, with 10 µl injection volume onto a Phenomenex Luna C18(2) column (150 X 4.0 mm i.d., 5 µm particle size) held at 30°C. A gradient elution was employed consisting of a mobile phase of 1% HCO_2H in MeOH (A) and 1% HCO_2H in H_2O (B) at a flow rate of 0.5 ml min^{-1} (A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min). For use as an analytical standard, several mg of GTX I were isolated from flowers of *R. ponticum* through process of solvent partitioning and prep-HPLC, adapted from previously reported methods (Elnaggar et al. 1980, Chen et al. 2004). A high (>95%) purity was confirmed; determined by ^{1}H and ^{13}C NMR and LC-MS, as described in (Tiedeken et al. 2014). While trace levels of other GTXs may exist in this species, we restricted our analysis to GTX I and GTX III, as these were by far the dominant GTXs. Calibration curves for GTX I and a commercial standard of GTX III (purchased from Sigma Aldrich; ≥90% purity), with quantification based on peak areas obtained from extracted ion chromatograms of the *m/z* 411.1 GTXI; Rt = 8.1 min) and *m/z* 415.3 (GTXIII; Rt = 6.71 min) molecular ions in negative electrospray ionisation mode, allowed the concentrations of compounds in nectar to be calculated.

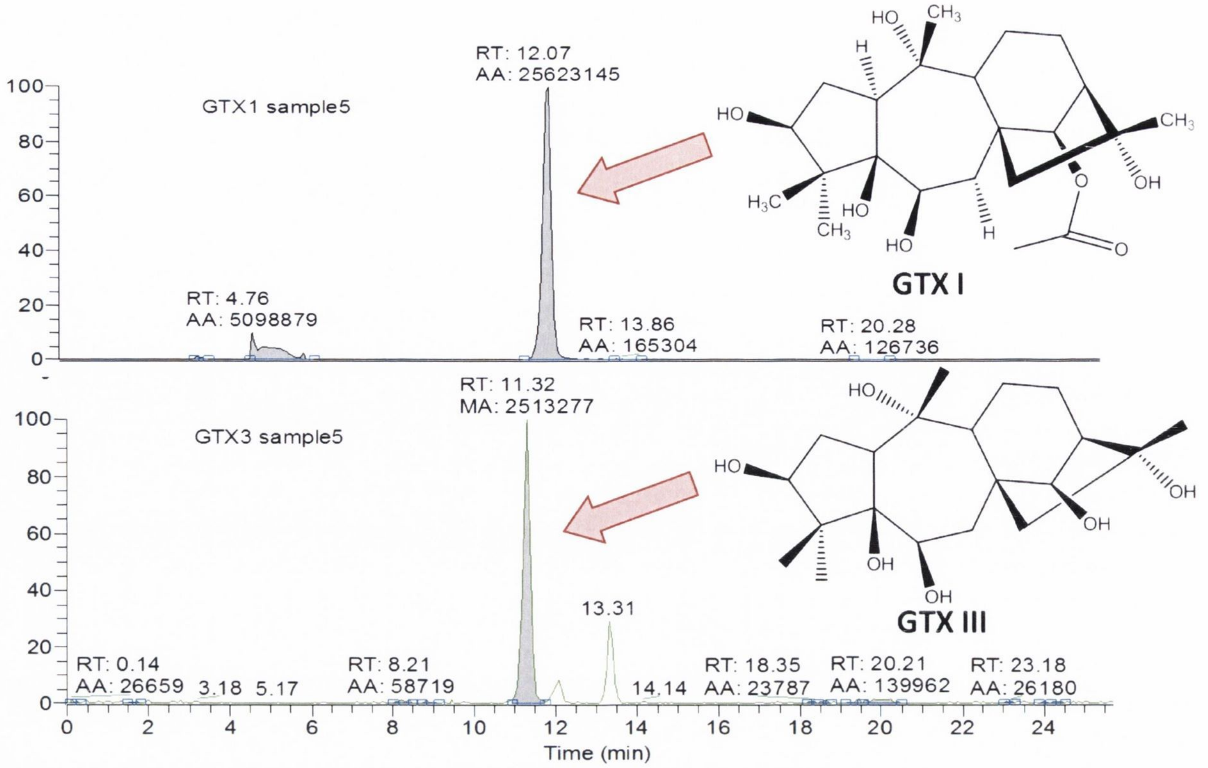

Figure 1. Chromatograms from LC-MS analysis of nectar representative of Chemotype A (GTX I-dominant), above; and Chemotype B (GTX III-dominant), below.

Final values of GTX were expressed as a concentration in dry weight of nectar (μg/mg dw), in order to account for environmental influences such as dilution or concentration of nectar. Dry and fresh weight concentrations were nonetheless highly correlated ($r^2 = 0.89$).

2.3.3 Data analyses

A linear mixed model (LMM) framework was employed to examine geographic variation and abiotic influences on nectar toxin levels. All models were fit by REML estimation in the nlme package (Pinheiro et al. 2013) in R version 3.0.1 (R Core Team, 2013), and validated through both statistical and graphical examination of standardised residuals for normality, homogeneity and independence, including spatial autocorrelation (Zuur et al. 2009). An information theoretic approach (AIC – Akaike Information Criterion) was used to select optimal model structure and assess relative performance. Model R^2 values were generated according to Nakagawa and Schielzeth (2013), for estimation of marginal ($R^2_{(m)}$) and conditional ($R^2_{(c)}$) variance explained by fixed effects and the total model, respectively. GTX I and GTX III values exhibited a Poisson distribution (due to the presence of legitimate zero values), and were square root transformed before analyses.

Geographic variation in quantitative and qualitative expression of GTX in nectar – To compare variation in GTX expression in the nectar of plants from different parts of *R. ponticum*'s geographic range, the dependant variables of nectar GTX (both GTX I & III concentration) and nectar water content were fit in LMMs against region (either Ireland, Spain, N Portugal or S. Portugal) as a fixed effect, and population nested within region as a random effect. Non-equal variance of residuals between regions was accounted for in the GTX III model by incorporation of a variance correlation structure (based on population identity), which significantly improved model AIC (Likelihood-ratio, *L* = 39.64, *df*= 6, *p*=<0.001). Post-hoc pairwise Tukey contrasts (corrected for multiple comparisons) were performed in the multcomp package (Hothorn et al. 2008) in R, in order to determine which regions differed significantly from each other, for the traits examined.

For qualitative comparisons of nectar toxins, the relationship between GTX I and GTX III concentration in the nectar of plant individuals was investigated through correlations (excluding cases with zero values for either compound), conducted separately for plants in the native (*n*=63) and invasive range (*n*=38). In addition, on the basis of the dominant GTX in nectar, plants were divided into two chemotypes; with those that produced abundant GTX I as the main toxin in nectar designated as Chemotype A, and plants which exhibited GTX III as the predominant nectar toxin (with little or no detectable GTX I) classified as Chemotype B (Fig. 1). The number and geographic distribution of plants expressing these distinct chemotypes was then investigated.

Within and between population spatial patterning of GTX expression – The extent of partitioning of variation in GTX I levels within and between populations was examined through LMMs, after accounting for abiotic sources of variation (as described in the following section). To examine spatial autocorrelation, mantel correlogram analyses were performed in the R package 'vegan' (Oksanen et al. 2013) to determine the similarity in GTX I concentration among plants and populations according to their spatial aggregation. Correlograms were calculated for distance lags of 24 m (for fine-scale within-population analysis), and 5-130 km (for broad-scale between-population analysis; as populations existed only at irregular distances from each other), with Holm's progressive correction for multiple-testing (*n*= 999 permutations).

Abiotic conditions and GTX expression – To investigate potential abiotic correlates of nectar GTX I concentration, a range of 16 microhabitat and landscape-scale variables (Table 1) were included as fixed effects in a spatially-explicit LMM. These variables were first assessed for multicolinearity by variance inflation factors (VIFs), and model simplification performed through evaluation of AIC and removal of non-significant terms (at an α-level of 0.05). Independence was violated in all models due to notable spatial autocorrelation of residuals, and was thus accounted for through specification of a spatial correlation structure (based on the coordinates of plants, recorded in the field with a GPS to an accuracy of 10 m) – which significantly improved AIC of the final optimal model (*L* = 6.52, *df*= 1,

p= 0.038). Additionally, mean nectar GTX I concentration per plant was compared between two years in population I2 using a paired *t*-test (see 'sampling locations').

2.4 Results

2.4.1 Geographic variation in quantitative and qualitative expression of GTX in nectar

GTXs were present in all nectar sampled from across the geographic range of *R. ponticum*. GTX I concentration was highest in the native Spanish populations (mean GTX I = 2.28 ± 0.19 μg/mg) compared with the invasive Irish populations (0.90 ± 0.11 μg/mg), but varied between regions within the native range (Fig. 2). In particular, nectar from plants in the Portugal South region contained very low concentrations of GTX I. In contrast to GTX I, other nectar traits including water content and GTX III levels remained relatively constant between the native and invasive range, and across native regions (Fig. 2).

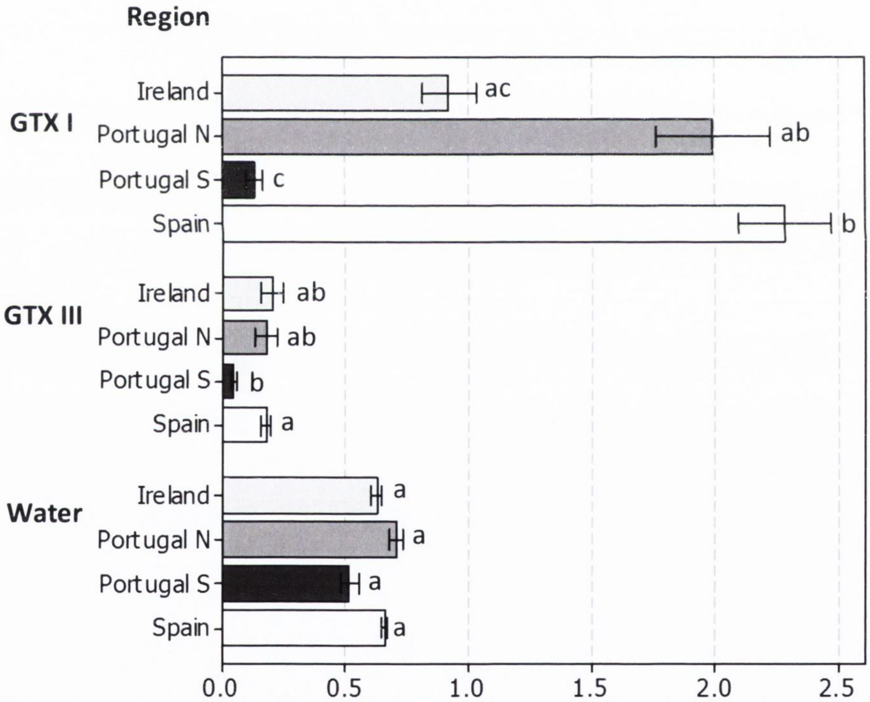

Figure 2. Comparison of nectar toxin levels (GTX I & III μg/mg dw ± SE) and nectar water content (mg/mg ± SE) between regions of *R. ponticum*. Linear mixed models were fit with region as a fixed effect and population as a nested random effect, accounting explicitly for unequal variance (sample sizes as listed in Appendix 1). The marginal and conditional R^2 for GTX I ($R^2_{(m)}$ =0.44, $R^2_{(c)}$ =0.63), GTX III ($R^2_{(m)}$ =0.14, $R^2_{(c)}$ =0.36), and nectar water content ($R^2_{(m)}$ =0.13, $R^2_{(c)}$ =0.45) indicated high levels of variation were explained by region, and population nested within region, respectively. Means which do not share a letter differ significantly, according to pairwise Tukey contrasts.

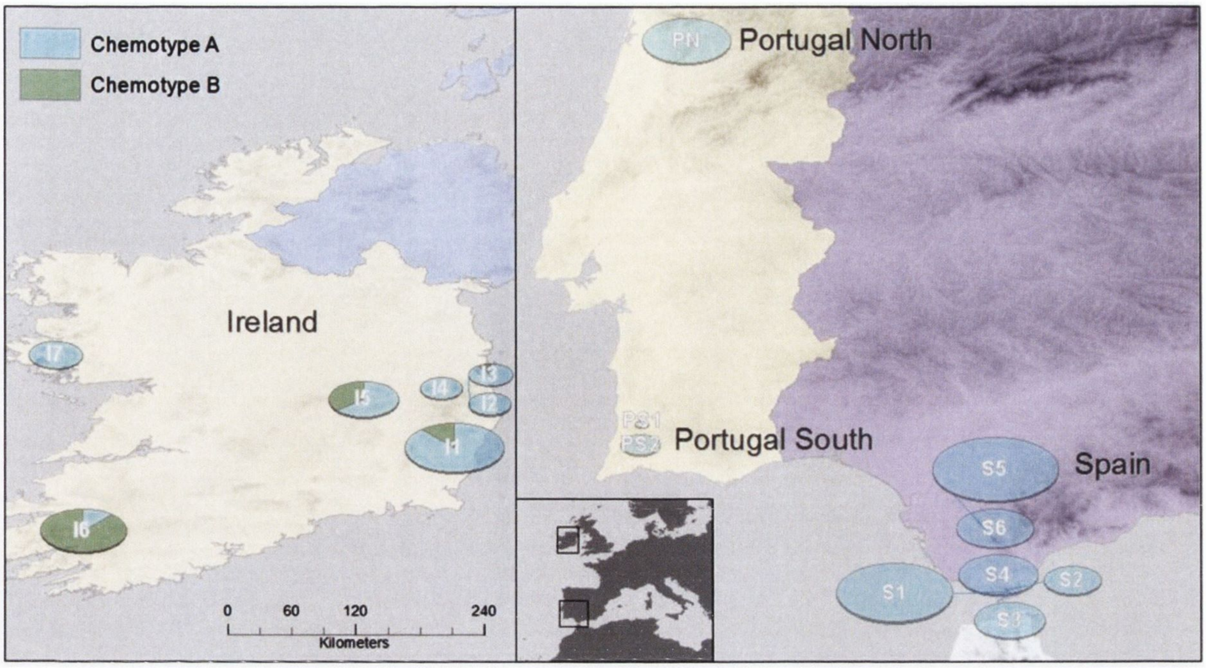

Figure 3. Spatial distribution of nectar chemotypes of *R. ponticum* within and between the native and invasive range. The proportion of plants exhibiting Chemotype A (GTX I-dominant) and Chemotype B (GTX III-dominant) is depicted per population, with pie-chart size proportional to mean GTX levels.

On a qualitative basis, a unique chemotype was evident throughout the majority of populations of the invasive range, which possessed GTX III as the main toxin in nectar (Figure 3). This 'GTX III-predominant' chemotype (i.e. Chemotype B) was absent amongst all plants examined in the native range (n=71), but constituted 22 % of plants in the invasive range (n=50). In the invasive range, ca. 18 % of these were completely devoid of GTX I in nectar. Thus while the concentration of GTX I and GTX III was linearly related in plants from the native range (r^2=0.66, p<0.001); this relationship was not evident in the invasive range (r^2=0.01, p=0.480), with the ratio of GTX I:GTX III on average 12:1 in the native range, and 3:1 in the invasive range.

2.4.2 Within and between population spatial patterns of GTX expression

In a LMM which incorporated both geographic and abiotic variation, a significant portion of variation in GTX I concentration was observed as location-specific, and partitioned mostly within rather than between populations (Table 2). Large gradients in nectar toxin concentrations were observed within populations, with an average five to six-fold range in concentration in the plants that produced GTX I. Individual plants and populations of *R. ponticum* demonstrated distinct patterns of spatial autocorrelation in GTX I content (Figure 4), which in both the native and invasive range was detected among individual plants within 70m of each other within populations (Figure 4a), and among populations within 15-23km of each other (Figure 4b). Thus only plants/populations which occurred beyond these distances were deemed independent of each other based on the characteristic of the amount of GTX I in nectar.

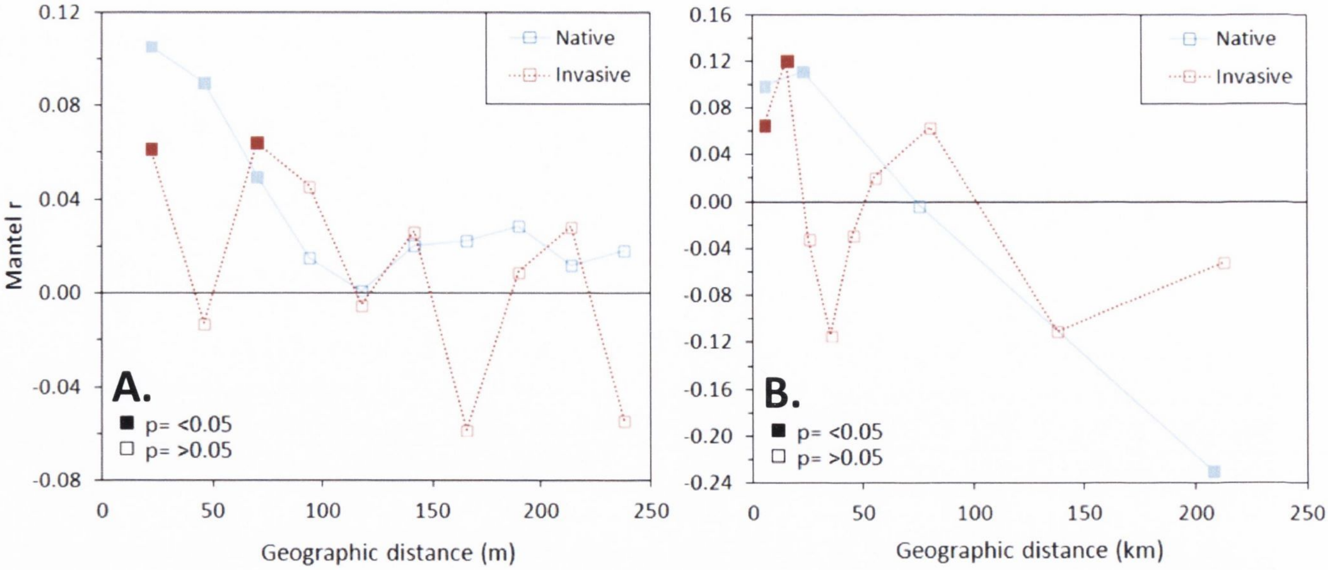

Figure 4. Mantel correlogram analysis of nectar GTX I content. Significant positive autocorrelation among plants was evident in both the native and invasive range at (A.) fine spatial scale up to 70 m, and (B.) at broad-scale up to 15-23 km. Spatial autocorrelation was absent or negative beyond these distances (with points close to the zero-line indicating non-significant Mantel r).

2.4.3 Abiotic conditions and GTX expression

Abiotic environment explained a significant amount of variation in nectar GTX I levels (Table 2). The model as a whole (accounting for both environmental and geographic attributes) successfully explained a high proportion of variation (R^2= 0.62). Attributes that varied at both microhabitat (canopy cover; aspect) and landscape (elevation; irradiance) scale were important, with higher GTX content associated with plants which grew in high light environments proximate to hotter SW aspects, but which retained significant levels of canopy shading. Nectar toxin concentration decreased as a function of elevation. Since these particular variables do not largely fluctuate on a year to year basis, it is not surprising that mean nectar toxin levels did not vary significantly between flowering seasons (paired t-test, *T*= -138, *p*= 0.217).

Table 2. Linear mixed model of nectar GTX I levels and its environmental association. Population is included as a random effect in the model, and spatial autocorrelation accounted for through addition of a spatial correlation structure; $N_{[populations]}$ = 16, $N_{[plants]}$ = 119. Beta coefficients indicate the relative importance and direction of influence of variables on GTX I.

Model[#]	**Beta coefficient** (± SE)	***t*-value**	***p*-value**	***R*²***
Fixed effects				0.37
Intercept	0.013 (± 0.139)	0.09	0.927	
Elevation	-0.427 (± 0.195)	-2.19	0.031	
Aspect (heat load)	0.155 (± 0.077)	2.03	0.045	
Irradiance	0.710 (± 0.179)	3.97	<0.001	
Canopy cover	0.189 (± 0.066)	2.85	0.005	
Random effects	Variance Component			
Between-population	0.074 (40%)	-	-	
Within-population	0.113 (60%)	-	-	
Total Model				0.62

[#] A likelihood-ratio test indicated the mixed model AIC was significantly lower than a null model containing random effects only (*L* = 6.58, *df*= 2, *p*= 0.037)

*Marginal ($R^2_{(m)}$) and conditional ($R^2_{(c)}$) variance explained for fixed effects and total model respectively

2.5 Discussion

2.5.1 *Variation and spatial pattern of nectar GTX expression*

Quantification of nectar toxins from throughout the geographic range of *R. ponticum* revealed high levels of GTXs in all plants from which nectar was sampled. With GTX I levels of up to 5.67 µg/mg (or 4110 µg/ml) found in Spanish plants, these concentrations represent some of the highest concentrations of toxic compounds known in nectar, according to comparison with published sources (London-Shafir et al. 2003, Adler and Irwin 2005, Boros et al. 2010, Adler and Irwin 2012, Manson et al. 2012, Cook et al. 2013, Gosselin et al. 2013). Concentrations of GTX I in *R. ponticum* nectar appear broadly similar to those reported in leaves and fresh twigs of this species (total GTX 9.28 µg/mg and 15.90 µg/mg respectively (Wong et al. 2002, Hough et al. 2010)), but are significantly more concentrated than levels found in 'mad honey' (GTX I 0.02 µg/mg honey) produced from flowers of the Turkish subspecies *R. ponticum* subsp. *ponticum* (Kurtoglu et al. 2014).

However, high intraspecific variation in GTX I concentration in nectar was revealed between the invasive and ancestral Spanish range of *R. ponticum* (Milne and Abbott 2000). There was also variation among Iberian regions; and in particular, plants in the Portugal South region had relatively low GTX I content. Although geographic variation in secondary compounds is common in many plant species (Levy 1983, Berenbaum and Zangerl 2006), including *Rhododendron* (Harborne 1986), no such studies have previously been available for secondary compounds in nectar. While high inter-population variability in nectar toxin content has previously been reported in *Nicotiana attenuata*

(Kessler et al. 2012), we have shown how variation may also be discretely partitioned over larger geographic scales.

In addition to discrete geographic variation, we assessed the spatial pattern of GTX expression in nectar on a continuous basis between neighbouring plants and populations. The finding of significant autocorrelation at both inter-plant (up to 70 m) and population (up to 15-23 km) scales may be explained by the fact that neighbouring plants/populations are more likely to share similar microhabitat or climatic conditions which may influence nectar toxin levels, and/or possess similar levels of expression due to their closer genetic relation (assuming a heritable basis to nectar GTX levels). Very few studies have examined how nectar traits are structured in a spatial context, despite evidence that autocorrelation at the patch level may facilitate the efficiency by which pollinators forage on plants displaying desirable nectar qualities (Leiss and Klinkhamer 2005). Spatial aggregation of low GTX-producing plants in both the native and invasive ranges of this plant could facilitate pollinator foraging, and/or the avoidance of plants expressing high levels of nectar GTXs. This may be important firstly, because it has been suggested that generalist pollinators of *R. ponticum* have poor acuity for the detection of nectar GTXs (Tiedeken et al. 2014), and secondly, due to the typically large gradients in nectar toxicity potentially encountered within populations (i.e. five to six-fold differences between plants).

2.5.2 Do environmental influences underpin geographic differentiation?

Four environmental variables examined (irradiance, elevation, aspect and canopy cover), which vary at local and regional scales, were associated with significant variation in nectar toxin levels. These variables influence the known niche of *R. ponticum* (Cross 1975a), and from interpretation of their specific magnitude and direction of influence on nectar toxin levels, it appears that plants which grew under more favourable conditions were associated with higher relative levels of GTX in nectar. This finding concurs with general predictions for carbon-based terpene secondary metabolites in woody plants (Koricheva et al. 1998), and in particular with a past investigation of diterpene expression in response to light levels (Bertolucci et al. 2013). Variation in GTX expression associated with microhabitat (i.e. canopy cover and aspect – which often varied over metres within populations) may represent a plastic response in plants, as opposed to more long-term genetic adaptation to abiotic conditions. Given the notoriously labile nature of floral nectar (Mitchell 2004, Canto et al. 2011), the temporal constancy in nectar toxin expression that we found across growing seasons is in contrast to reports for other traits such as nectar volume – which, in a suspected toxic-nectar producing species of the closely related genus *Kalmia* L. (Howes 1949), varied largely in individuals between years (Real and Rathcke 1991).

While environmental conditions may significantly influence nectar GTX levels in plants, it is unlikely that these alone could account for the large quantitative reduction in GTX I concentration (and the widespread occurrence of a unique chemotype devoid of GTX I) observed between the native Spanish and invasive range. Moreover, given that it was only GTX I which changed in concentration in invasive plants, it is difficult to conceive how abiotic conditions could drastically alter expression of this compound to this extent, while bearing no influence on GTX III – a structurally similar, biosynthetically related GTX (Qiang et al. 2011) – as well as other nectar traits. Random genetic drift and founder effects also do not appear as a likely cause; given the magnitude of change specific to GTX I, and the fact that Irish invasive populations retain levels of genetic and morphological diversity comparable to that found in the ancestral range (Erfmeier and Bruelheide 2011, Stout et al. 2015). Instead, natural selection in response to biotic pressures (which perhaps target enzymes required to produce or transport GTX I into nectar) appears to offer the most likely explanation. Given that there are considerable metabolic costs to the production of plant terpenoids (Gershenzon 1994), both enemy-release from antagonist herbivores (including nectar robbers), or pollinator-mediated selection against high nectar toxin levels, offer conceivable hypotheses as to why invasive plants of *R. ponticum* show reduced nectar toxin levels. Testing whether directional selection for reduced nectar toxicity is apparent within the invasive range may therefore provide greater insight into these particular questions.

Chapter 3

Mutualisms win the day: pollinators rather than herbivores mediate selection on a plant chemical defence

"Nothing in biology makes sense except in the light of evolution".

– Theodosius Dobzhansky

3 Mutualisms win the day: pollinators rather than herbivores mediate selection on a plant chemical defence

3.1 Abstract

To-date, studies of natural selection on plant chemical defence traits have almost exclusively focused on plant-antagonist interactions. However, plant chemical defences may also impact mutualists such as pollinators or frugivores due to expression in rewards such as fruit, pollen and nectar. Here, I examined multispecies interactions in the invasive plant species *Rhododendron ponticum* (Ericaceae), which constitutively expresses diterpene grayanotoxins (GTXs) throughout most plant tissue types, including nectar. Assessment of the spatial pattern and magnitude of selection on toxin levels in *R. ponticum* revealed positive directional selection on GTX levels in leaves, flowers and nectar in the species' native range, whereas in the invasive range nectar GTX levels experienced negative selection. In investigating biotic pressures potentially driving natural selection, selection on leaf toxin levels was consistent with the finding that GTX I conferred resistance against a generalist herbivore of this species, whereas pollinator-mediated selection was indicated by the higher intensity of pollen limitation experienced in plants exhibiting high nectar toxin levels. This study therefore represents the first empirical evidence of pollinator-mediated selection on a plant chemical defence. As a whole, the generality of these findings in nature may in fact be broad, given the relatively widespread occurrence of toxic nectar amongst plant families, and where species are distributed over large ranges throughout which diffuse selective pressures from antagonists or mutualists may occur.

3.2 Introduction

Plants must simultaneously interact with a diverse assemblage of mutualist and antagonist species which require attracting or defending against (Strauss 1997, Kessler and Halitschke 2009). Yet despite the ubiquitous abundance of multi-species interactions in natural systems, most investigations show an overwhelming bias towards simple pairwise interactions – such as within plant-pollinator or plant-herbivore systems (Herrera et al. 2002). While a pair-wise approach will continue to generate important insights, growing interest in multispecies interactions has allowed a deeper understanding of how plants cope with and adapt to multiple – often opposing – selective pressures in both ecological and evolutionary terms (Irwin et al. 2004, Strauss and Irwin 2004, Irwin and Adler 2006). The best known examples in this regard involve conflicting pressures imposed by herbivores and pollinators on plant apparency and associated visual and chemical attraction cues (Euler and Baldwin 1996, Galen and Cuba 2001, Theis et al. 2007, Halitschke et al. 2008, Lay et al.

2011); the outcome of which in natural populations can enhance phenotypic variation or result in temporally fluctuating levels of trait expression (Strauss and Irwin 2004, Ågren et al. 2013). However, where the impact of antagonists bears large fitness consequences due to altered plant mutualisms, optimal trade-offs cannot always be easily reached – for example, where foliar or floral herbivory negatively impacts pollinator visitation due to damage to attractive structures (Lohman et al. 1996, McCall and Irwin 2006) or the induction of defensive compounds in floral rewards (Adler et al. 2001, Adler et al. 2006, Kessler and Halitschke 2009). As such, it has typically been shown that selection imposed by herbivores on plant traits generally overwhelms that which is pollinator-mediated (Cariveau et al. 2004, Irwin 2006, Strauss and Whittall 2006, Gomez et al. 2009). Whereas in contrast, it is apparently rare, or rarely detected, that pollinators impose stronger net selection on traits of shared relevance to herbivores (Bartkowska and Johnston 2012).

To-date, studies of natural selection on plant chemical defence traits have almost exclusively focused on plant-antagonist interactions (Ibanez et al. 2012) – despite the fact that plant chemical defences may also impact mutualists such as pollinators or frugivores due to expression in rewards such as fruit, pollen and nectar (Cook et al. 2013, Arnold et al. 2014, Whitehead and Bowers 2014). In particular, the expression of chemicals usually associated with defence in nectar, the most important mediator of interactions with mutualists (Heil 2011), appears paradoxical, but is none-the-less widespread throughout many plant families (Baker 1977, Adler 2000). While a number of hypothesized adaptive or maladaptive functions of so-called toxic nectar are currently supported (Adler and Irwin 2005, Gegear et al. 2007, Manson et al. 2010, Kessler et al. 2012, Wright et al. 2013), direct pollinator-mediated selection in natural populations either for or against toxic nectar has not been empirically demonstrated. Here, we investigated a potential diffuse scenario of natural selection on toxin expression in the invasive plant species *Rhododendron ponticum* (Ericaceae). Species of this genus are known to constitutively express diterpene grayanotoxins (GTXs) throughout most plant tissue types as a potent chemical defence; in *R. ponticum* this also includes nectar (Chapter 2). Our past work on this model system has shown that while some pollinators tolerate this toxic nectar, others do not (Tiedeken 2015). The observation that a predominant nectar toxin (GTX I) is absent or significantly reduced in invasive plants leads us to question if this pattern is due to pollinator-mediated selection (Chapter 2). However, as GTXs are also known to serve as highly effective herbivore antifeedants in plants (Klocke et al. 1991), there exists the potential for conflicting pressures on this trait from both mutualist and antagonist sources.

We assessed natural selection on leaf, nectar and flower toxin levels within both the native and invasive range of *R. ponticum* through a path analysis framework, backed by observational and experimental tests of causation from biotic agents. Path analysis provides a useful extension to multiple regression analysis of natural selection (Lande and Arnold 1983, Scheiner et al. 2000) and offers an ideal solution to disentangling the fitness effects of tissues phenotypically correlated in their expression of chemical defence; which is frequently inferred for example between leaves, phloem and nectar (Adler et al. 2006, Manson et al. 2012, Lohaus and Schwerdtfeger 2014). Based on this general observation and biosynthetic evidence specific to GTXs (Masutani et al. 1981, Bouvier et al. 2005), we devised path models reflecting a main foliar-plastid biogenesis of toxins leading to expression in flowers and nectar. The main goals of this study therefore were to investigate the spatial pattern and magnitude of selection on toxin levels in nectar, leaves and flowers of *R. ponticum*, and assess whether these selection regimes; A.) can explain the observed phenotypic divergence in nectar toxin levels between ranges, as previously revealed (Chapter 2); and B.) are consistent with biotic selective pressures as the main drivers of these – as evaluated by field observations and experimental manipulation of pollen limitation and plant resistance to herbivory.

3.3 Methods

3.3.1 Location, traits and abiotic variables measured

The study was conducted in southern Spain and northern and southern Portugal in nine native populations of *R. ponticum* (i.e. *R.p.* subsp. *baeticum*), and four populations in the species' invasive range in Ireland (Appendix 1). Between six and ten plants per population were sampled, with a minimum distance of 20 m between individuals to reduce the chances of sampling ramets. For each individual, nectar, leaf and corolla material was collected, and floral morphological and abiotic variables quantified. Nectar was collected from between 8-15 flowers (pooled until ca. 1 µl was obtained for each individual) using microcapillary tubes, after which the corolla and nearest sub-tending leaf were removed and immersed in silica gel in snap-seal bags. Care was taken not to damage floral tissue until after nectar samples were collected. In the lab, water content was removed by freeze drying nectar and oven drying leaf and flower samples, and material then pooled to produce one composite sample per plant for each tissue type. Dried flower and leaf samples (30 mg) were ground to a homogenous powder and extracted (3 X 20 ml) in MeOH, and a 200 µl aliquot transferred to analysis vials. Dried nectar was re-suspended in 200 µl MeOH for analysis. Quantification of GTX I was carried out by LC-MS analysis as previously detailed (Chapter 2). Final values of GTX I were expressed as a concentration of dry weight of tissue (µg/mg dw). Mean corolla width (measured as the widest horizontal distance between the tips of petal wings) and corolla tube width (measured as the internal diameter of the corolla tube at its base) were recorded with dial callipers from five flowers per plant. We previously reported that several microhabitat factors (canopy cover, aspect, elevation and irradiance) explained a significant amount of variation in nectar toxin levels in *R. ponticum* (Chapter 2). Where appropriate, we utilised these variables to control for the confounding effect of environmental heterogeneity in models featuring toxin levels as an explanatory variable.

3.3.2 Quantification of relative fitness and pollen limitation

Maternal fitness was measured as total seed set per plant. Calculation of seed set in tall, profusely flowering shrub or tree species can prove challenging, and hence a sub-sampling approach is often employed (Gomez 2004, Sobral et al. 2010). To obtain estimates of total seed set in *R. ponticum* plants, we first calculated mean seed set from 8-40 capsules (depending on flower abundance per plant) using a precise equation to estimate seed number based on measurement of mature capsule length (Mejías et al. 2002, Stout 2007b). To estimate the total number of flowers per plant, we counted the number of flower trusses (racemes consisting of a pseudo-whorl of usually 9-12 flowers) per individual and multiplied this by the mean flower number (inclusive of those at pre and post-anthesis stage) obtained from 15-20 trusses. Although not all flowers mature into fruiting capsules, these measures are none-the-less highly correlated in *R. ponticum* (Mejías et al. 2002). We then multiplied total number of flowers per plant by the mean seed set per capsule to afford total seed set. Finally, relative fitness was calculated by dividing individual seed set by the range mean.

A cohort of five individuals per population was selected for application of a supplementary pollination treatment to measure pollen limitation. Although *R. ponticum* is self-fertile, optimal seed set occurs under xenogamy (Stout 2007b), and in particular due to intrapopulation cross-pollination (Mejías et al. 2002). The supplemental treatment thus consisted of application of recently dehisced anthers from neighbouring plants (≥ 35 m distance away) to receptive stigmas of target flowers, ensuring deposition of long viscin pollen threads. Treated flowers were tagged and collected just preceding capsule dehiscence (mid-October in the native range; late January in the invasive range), with an overall retrieval rate of 88 % (due to wind damage, natural excision etc.). Pollen limitation was thus assumed in plants where supplementally-treated flowers exhibited significantly higher seed set than open-pollinated flowers, according to one-tailed Welch's *t*-tests. The resultant *t*-value of this test was taken as a continuous, quantitative measure of the magnitude of pollen limitation per plant. While the ability to differentially allocate resources to out-crossed flowers has been noted in some species (Ashman et al. 2004, Wesselingh 2007), we did not believe this to be a confounding issue in our measure of pollen limitation given the large gradients and spatially consistent patterns which were subsequently observed.

3.3.3 Field and experimental assessment of resistance to herbivory

All plants from which traits were measured were in addition surveyed for herbivore damage. Due to the typically large size of shrubs, we assessed 1 m^3 areas at the edge of individuals from ground level upwards. The total number of young and old leaves within this area was counted, and the number of leaves exhibiting herbivore damage recorded for each age class. If present, the area of damage on leaves was usually consistent (ca. 10-15 % area removed). Three generalist species of broad-nosed weevil (Coleoptera: Curculionidae: Entiminae) known to feed on *R. ponticum* in the invasive range (Judd and Rotherham 1992, Yela and Lawton 1997), the Nut leaf weevil (*Strophosoma melanogrammum* Forster), Clay-coloured weevil (*Otiorhynchus singularis* L.) and Black vine weevil (*Otiorhynchus sulcatus* Fabricius), were selected for experiments. Wild adult weevils of the former two species were collected from populations of *R. ponticum*, while the latter was reared from larval stage in a glasshouse on strawberry plants. However, as Nut leaf and Clay-coloured weevils refused to feed on artificial diets (see below) during initial trials, further experiments with these species were not attempted. Bioassays with black vine weevils (BVWs) were conducted using late instar adults in pre-oviposition period; a phase lasting 3-6 weeks during which time they consume the most plant foliage. Thirty adults were placed into individual arenas (20 X 10 X 6 cm) and randomly allocated to three treatments: 1.) a control artificial diet; 2.) an artificial diet in which GTX I was incorporated at natural leaf concentrations; and 3.) an artificial diet in which ten times the natural concentrations of GTX I was incorporated. Artificial diets for BVWs were constructed following established techniques (Bristow et al. 1979, Shanks and Doss 1987), which consisted of cellulose acetate disks (0.45 µm pore size) treated with water-dissolved sucrose and β-sitosterol phagostimulants at concentrations known to solicit high feeding rates (Doss and Shanks 1984). Sample sizes (the number of BVWs per treatment) were constrained by the limited quantity of GTX I we were able to isolate from several kg of dried *R. ponticum* flowers, as per methods previously reported (Tiedeken et al. 2014). However, since there is typically low between-individual variation in BVWs due to obligate parthenogenesis (Stenberg and Lundmark 2004), we considered these sample sizes adequate. Experiments were conducted for a total of 11 days (with a single change of cellulose disks at day 5.5) in conditions maintained at ca. 21 °C and 85 % relative humidity (Shanks and Finnigan 1973, Fisher and Bruck 2004). The cumulative area eaten (mm^2) from disks was quantified per weevil from digital scans using ImageJ analysis software (National Institutes of Health, Bethesda, Maryland, USA). For both field and laboratory assessments, results are reported in terms of resistance (i.e. 1 minus % herbivore damage). In addition to resistance, we also assessed tolerance to herbivory as a complementary defence strategy which is often co-employed by plants (Carmona and Fornoni 2013). In field studies, tolerance was quantified per range as the slope of relative fitness regressed on percent herbivore damage (Wise and Carr 2008).

3.3.4 Data analysis

Comparison of trait means across ranges – Geographic variation in GTX I levels was analysed per tissue type in linear mixed models (LMMs) fit by restricted maximum likelihood (REML) estimation in the R package nlme (Pinheiro et al. 2013). As invasive plants are known to have originated from

Spanish as opposed to disjunct Portuguese populations (Milne and Abbott 2000), we restricted native-invasive range comparisons to the former only. Nectar, leaf and flower GTX I levels were square root transformed (to improve normality) and fit in LMMs as dependant variables against range (native or invasive) as a fixed effect and population as a nested random effect – with microhabitat variables included as covariates. For model validation, standardised residuals were examined for normality, homogeneity and independence, including spatial autocorrelation (Zuur et al. 2009). Non-equal variance of residuals between populations was accounted for in the leaf GTX I model by incorporation of a variance correlation structure (based on population identity), which significantly improved model AIC (Likelihood-ratio test; $L = 20.3$, $p=0.016$). As three individual LMMs were conducted comparing tissue toxin content between ranges, we employed Benjamini-Hochberg adjustment of *p*-values in order to reduce the familywise error rate.

Natural selection on plant toxin levels – Preceding selection analyses, we first: A.) controlled traits for potential confounding effects of environmental heterogeneity, as strong abiotic-mediated covariance between traits and fitness can bias estimates of selection gradients (Rausher 1992, Ashman and Morgan 2004); and B.) affirmed the legitimacy of pooling population data (Scheiner et al. 2000), for analysing selection at the range level. Details of these steps are provided in supplementary methods (Appendix 3). Subsequently, estimates of directional selection were obtained for each range through multiple regression of relative fitness on standardized traits (Lande and Arnold 1983) within a path analytical framework – following terminology of Scheiner et al. (2000). For path models, a hypothesized causal structure between leaf, nectar and flower GTX I levels and relative fitness was assessed. In addition, we tested for non-linear selection on traits, including quadratic (disruptive/stabilizing) and correlational selection (Lande and Arnold 1983). However, as no significant non-linear selection was detected (data not shown), we focussed on directional selection only. We employed mean-standardization of traits to allow output of mean-standardized selection gradients (β_μ) from analyses, as a measure of intensity of selection. These are deemed superior where comparisons of the strength of natural selection are desired, for instance between traits, or across geographic space (Matsumura et al. 2012) – with the added advantage of their interpretation as fitness elasticities (van Tienderen 2000, Hereford et al. 2004); the resultant change in relative fitness from doubling trait values.

Path and mediation analyses were carried out using the R package 'lavaan' (Rosseel 2012) for structural equation modelling. Data for both ranges were assessed for multivariate normality by Mardia's test in the R package 'MVN' (Korkmaz and Goksuluk 2014), and as neither dataset met this requirement, we opted for robust maximum likelihood estimation of path coefficients as a non-parametric alternative. Path model goodness-of-fit is reported as the Satorra-Bentler adjusted Chisquared (χ^2), for better approximation of *p*-values under non-normality. Subsequent to estimation of path coefficients, mediation analysis was employed to test the significance in path models of direct selection gradients (β_μ) (assessed along forward-connected paths from a trait to fitness, inclusive of any mediation through intermediate traits); indirect selection (assessed as paths which lead forward to fitness first through a backwards step), and total selection differentials (denoted *s*; the sum of direct and indirect selection) (Kingsolver and Schemske 1991, Scheiner et al. 2000). Selection differentials estimated within a path model are also referred to as the 'predicted covariance', as values will usually differ from as typically measured (i.e. through simple trait-fitness correlations) in the absence of causal structure (Scheiner et al. 2000, Cohen et al. 2013). As fitness measurements were not taken and/or could not be retrieved on all plants, missing values were casewise deleted. Final sample sizes for path and mediation analyses were *N*= 68 (i.e. *N*= 38 for the native range; *N*= 30 for the invasive range); 45-60 % of the median sample size reported for plants in a systematic review on selection (Siepielski et al. 2013), and at least ten times the number of model explanatory variables, as per standard guidelines (Mitchell 2001).

Biotic selection pressures on plant toxin levels – Differences in both the frequency and intensity of pollen limitation between ranges were assessed through Pearson's Chi-square Test for Independence and by t-test, respectively. Following this, multiple regression analyses were conducted for each range, to examine potential biotic and abiotic determinants of pollination limitation. In addition to nectar toxins, a range of floral morphological (corolla and tube width) and microhabitat variables (canopy cover, aspect, elevation and irradiance) were considered for inclusion in models as potential co-determinants, while monitoring multicollinearity through variable inflation factors (VIFs). Final regression models contained explanatory variables significant after Benjamini-Hochberg adjustment of *p*-values. A Generalised Linear Model (GLM) with quasi-binomial errors (to account for overdispersed proportional data) was used to determine if there were differences in resistance to BVW among GTX I treatments (control, normal, x10). Post-hoc Tukey pairwise comparisons were used to determine which treatments were significantly different from one another, using the R package multcomp (Hothorn et al. 2008)), and corrected for multiple comparisons by Benjamini-Hochberg adjustment. The frequency of herbivore damage on plants in the field was analysed according to the factors of leaf age and range of provenance (i.e. native or invasive) using Pearson's Chi-square Test for Independence. To investigate whether observed levels of plant resistance (i.e. 1 minus % herbivore damage) in the field could be explained by leaf GTX I and other microhabitat variables (as listed above) we fitted GLMs for each range with a quasi-binomial distribution (to account for over-dispersion). The overall significance of GLMs was assessed through comparison with a null model, and McFadden's pseudo-R^2 generated to assess model fit.

3.4 Results

3.4.1 Natural selection on plant toxin levels

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

Decomposition of total selection on traits into direct and indirect components revealed that total selection on nectar and flower toxin levels in the native range is the result of large indirect selection acting through leaves (Table 1), while in the invasive range, only direct selection on nectar toxin levels was observed. Within ranges, no instances of conflicting selection on traits were observed, as indicated by the relative sign of coefficients (Table 1). Interpretation of mean-standardized direct selection gradients (β_μ) in Table 1 as fitness elasticities reveals that, for instance, a two thirds increase in mean leaf toxin levels in the native range would result in a 21.6 % increase in relative fitness, whereas a two thirds reduction in mean nectar toxin levels in the invasive range would result in a 10.8 % increase in relative fitness.

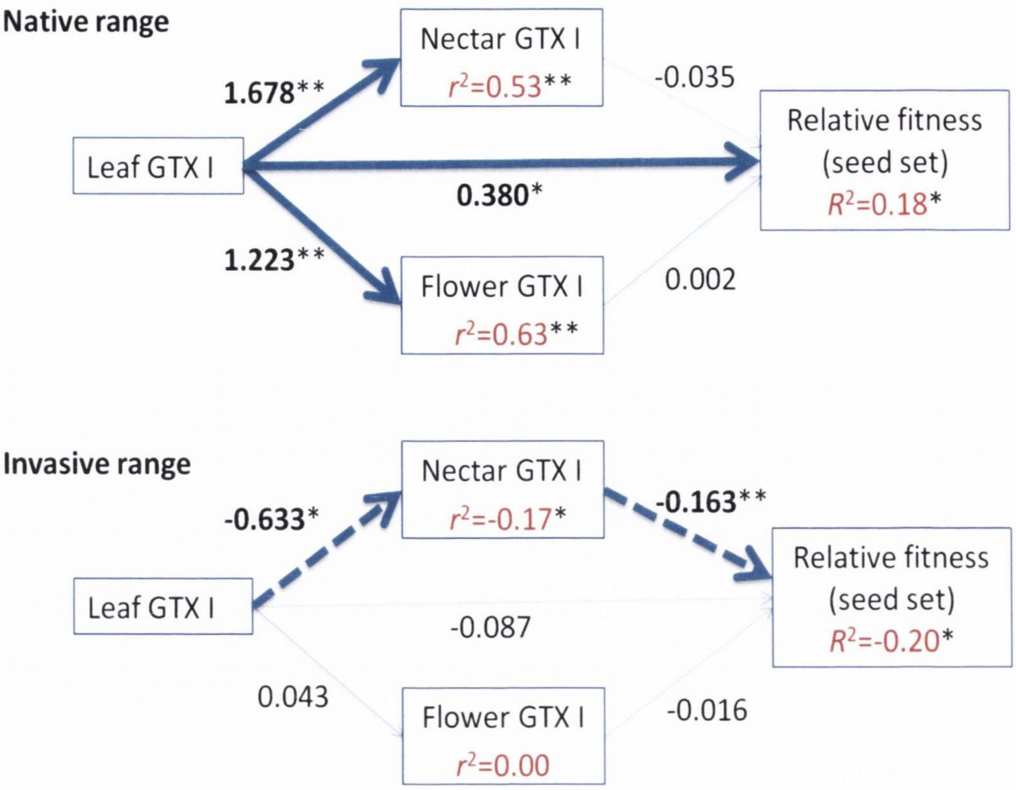

Figure 1. Solved elasticity path diagrams for directional selection on traits in the native (top) and invasive (bottom) range of *R. ponticum*. Mean-standardized path coefficients are presented, with dashed lines representing negative coefficients, and arrow width indicative of the strength of effect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

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

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

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

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

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

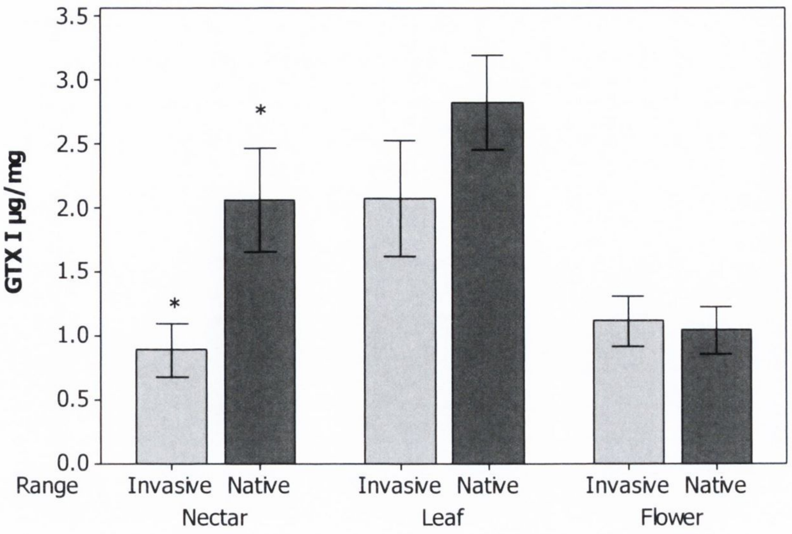

Figure 2. Mean tissue toxin levels (GTX I µg/mg ± 95% CI) in the native and invasive range of *R. ponticum*. For each tissue type, LMMs were fit with 'range' as a fixed effect and 'population' as a nested random effect, and were controlled for abiotic environment. After adjustment for multiple comparisons,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

3.4.2 Biotic selection pressures on plant toxin levels

Pollinator selection pressures – A significant difference was found between the native and invasive range in the frequency of plants exhibiting pollination limitation (χ^2 = 17.6, df = 1, p= ≤0.001); with seed set in 76 % of plants in the native range found to be pollen limited, compared to only 15 % of plants in the native range. In addition to frequency, there was also a difference in the intensity of pollen limitation in plants between the native (mean = 0.95 ±0.34) and invasive range (mean = 4.24 ±0.57) (t-test: t= 5.22, df= 44, p= ≤0.001). Subsequently, we examined a range of intrinsic biotic and extrinsic abiotic factors to determine potential causes of pollination limitation in each range. The same pattern was observed within both ranges, in that plants which were highly pollen limited possessed high levels of nectar toxins and also wider flower corollas (Table 3). Consistent with differences in the intensity of pollen limitation between ranges, however, the relative effect of nectar toxin content on pollen limitation was more than three times greater in the invasive than the native range.

Herbivore selection pressures – Evidence from controlled feeding experiments utilising an ecologically relevant herbivore of *R. ponticum* indicated that leaf GTX I functions as an important chemical defence conferring resistance to herbivory (Fig. 3a). Field observations in both the native and invasive range corroborated this finding, in that herbivory levels varied according to leaf age (χ^2 = 1159.2, df = 1, p= ≤0.001), with younger leaves exhibiting higher levels of damage. This may be

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

Range	**Coefficient** (± SE)	***t-value***	***p*-value**
Native†			
Nectar GTX I (µg/mg)	0.58 (±0.23)	2.52	0.020
Flower corolla width (mm)	0.22 (±0.07)	3.03	0.012
Invasive*			
Nectar GTX I (µg/mg)	1.83 (±0.76)	2.41	0.027
Flower corolla width (mm)	0.32 (±0.14)	2.24	0.039

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

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

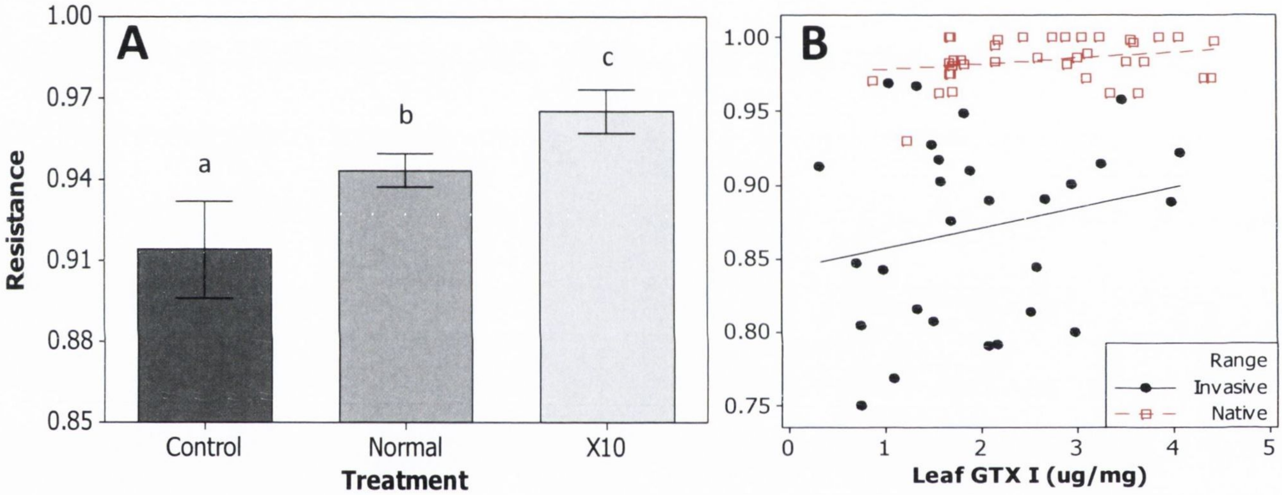

Figure 3. (A.) resistance to Black vine weevil (*Otiorhynchus sulcatus*) feeding as conferred by GTX I (mean ± SE). Treatments represent artificial diets in which GTX I was absent (Control), or incorporated at average leaf levels (Normal), or ten times this amount (X10). Means which do not share a letter differ significantly (at $p \leq 0.05$) according to one-tailed Tukey pairwise contrasts (corrected for multiple comparisons); and (B.) the relationship between leaf GTX I levels and resistance of plants to herbivory in the wild. A non-significant 'Range X Leaf GTX I' interaction revealed equivalency in the form of relationship between ranges (ANCOVA homogeneity of slopes: *df*= 61, *p*= >0.05).

explained by the fact that that young leaves contained significantly less GTX I than older leaves (Paired t-test: *t*= 4.05, *df*= 36, *p*= ≤0.001). However, although the form of the relationship did not differ significantly between ranges (Fig 3b); the ecological value of leaf GTX I in conferring resistance was not consistent across ranges, with herbivore damage to plants much more prevalent in the invasive rather than native range (χ^2 = 2181.8, *df* = 1, *p*= ≤0.001). Hence, in the invasive range a significant association was observed between resistance and leaf GTX I levels (*p*= 0.008) together with canopy cover (*p*= 0.014) (quasi-binomial GLM: $F_{2,29}$= 5.2, *p*= 0.014, pseudo R^2= 0.30); while neither of these variables were significant in the native range (quasi-binomial GLM: $F_{2,37}$= 0.4, p= 0.700, pseudo R^2= 0.02). The tolerance of plants to herbivory in both the native (slope = 0.010 ± 0.022) and invasive range (slope = -0.006 ± 0.007) was substantial, as neither slope differed significantly from zero (at an α-level of 0.05).

3.5 Discussion

This study aimed to evaluate natural selection on a toxic plant chemical defence across the native and non-native range of an invasive species, for which potentially conflicting selection pressures are exerted by mutualists and antagonists. Within ranges, no instances of conflicting selection between tissue types were observed. In the native range, positive total selection on toxin levels in flowers and nectar was as of an indirect result of selection on leaves; whereas in the invasive range nectar toxin levels experienced negative selection, while other traits were selectively neutral. However, in comparing selection regimes between ranges, we detected significant divergent selection acting on nectar toxin levels, which in addition matched phenotypic digression in mean trait levels observed in invasive plants. While other mechanisms may have in addition contributed to this difference in mean nectar toxin levels between ranges (as discussed in Chapter 2), the relative specificity and extent of targeted change to nectar toxin levels in comparison to other tissues, which is in addition restricted to the major nectar toxin GTX I (and not GTX III (Chapter 2)), provides evidence consistent with adaptive microevolution through natural selection.

While theory predicts the uncoupling of inter-correlated expression of secondary compounds across tissue types in response to sustained opposing selection pressures (McCall and Irwin 2006, Kessler and Halitschke 2009), such scenarios have seldom been tested or demonstrated in plants (Manson et al. 2012). We here provided evidence as to the occurrence of such a functional de-coupling, allowing invasive plants to have surmounted the ecological and evolutionary constraint posed by non-tissue specific expression of defensive compounds. Far from a mere up-loading of phloem constitutions, the production of floral nectar in plants represents a complex, multi-stage process involving transport or *de novo* synthesis of components across various functional nectary ultrastructures (Kram and Carter 2009, Heil 2011 and references therein). These may hence form legitimate targets for adaptive modification of nectar. A seemingly novel physiological adaptation within invasive plants affording independent regulation of nectar toxins has therefore permitted natural selection to act directly on this trait, while not reducing chemical defence in other tissues, and may thus have functioned as a key innovation permitting the still-expanding spread of this invasive species after two and a half centuries (Cross 1982). In contrast, due to inter-correlated linkage between tissue types in native plants, positive selection on leaf toxin levels resulted in large indirect selection on nectar toxin levels.

Investigation of biotic selection pressures in both ranges revealed that plants which exhibited high nectar GTX I levels also experienced higher intensities of pollen limitation. The vast majority of individuals in the invasive range were pollen-limited (compared to just 15 % in the native range) indicating that nectar toxin levels were here subject to negative pollinator-mediated selection; given the apparent importance of pollinators as selective-agents via seed production when plants are pollen-limited (Johnston 1991, Ashman and Morgan 2004, Bartkowska and Johnston 2012). That pollinator-mediated selection is, at least in part, actively exerted is evidenced in the finding that toxic nectar from *R. ponticum* can be highly lethal to honeybees in the invasive range, whom here mostly avoid it (Tiedeken 2015); whereas honeybees in the native range otherwise provide significant pollination services for *R. ponticum*, as evidenced by their production of 'mad' honey (Kurtoglu et al. 2014). Furthermore, due to demonstrated sublethal post-ingestive effects (Tiedeken 2015), established pollinators in the invasive range such as solitary bees may be differentially deterred by plant individuals on the basis of nectar toxicity. Preferential foraging behaviour in pollinators could be facilitated by the fact that low toxin producing plants tend to be spatially aggregated at the patch-level within populations (Chapter 2).

Positive selection was observed on leaf toxin levels in the native range, consistent with the finding that GTX I conferred resistance against a generalist herbivore of this species, and that young leaves with lower toxin levels exhibited greater relative damage throughout wild populations in both ranges. However, while there was a similarity between ranges in the general form of the relationship between leaf GTX I levels and resistance to herbivore damage, this relationship was only significant in the invasive range, where *R. ponticum* has experienced a notable gain in levels of herbivore damage. This scenario represents a seemingly rare contradiction (Maron and Vila 2001, Litt et al. 2014) of the enemy-release hypothesis, which is often invoked to explain the success of non-native species. Hence, explanation as to the somewhat counter-intuitive finding that leaf toxin levels were under positive selection in the native range, but not so in the invasive range, may therefore relate to the existence of other unmeasured relevant sources of herbivory, and the observed high level of tolerance employed as a complementary defence strategy by this species, respectively.

3.5.1 Conclusions

Where interactions involving mutualists and antagonists are mediated by the same trait in plants, rarely are pollinators implicated as predominate selective agents. This study represents the first empirical evidence of pollinator-mediated selection on a plant chemical defence; the adaptive significance of which is usually exclusively discussed within context of plant-herbivore interactions. These results also demonstrate how the divisive adaptation of nectar by plants – which is generally held as the most important mediator of interactions with mutualists – may facilitate colonization of exotic habitat, such as occurs in the invasion process. We recommend that future assessments of selection on chemical defence of multiple tissue types take into account the specific structure of phenotypic correlation amongst these; as ignoring such causal relationships, or assessment of single tissue types in isolation, may lead to largely differing conclusions. As a whole, the generality of these findings in nature may in fact be broad, given the relatively widespread occurrence of toxic nectar amongst plant families, and where species are distributed over large ranges throughout which diffuse selective pressures from antagonists or mutualists may occur.

Chapter 4

Did loss of toxic nectar accompany a shift in pollination syndromes within *Rhododendron*?

"Things exist either because they have recently come into existence or because they have qualities that made them unlikely to be destroyed in the past."

– Richard Dawkins

"Those who do not move, do not notice their chains."

– Rosa Luxemburg

4 Did loss of toxic nectar accompany a shift in pollination syndromes within *Rhododendron*?

4.1 Abstract

Toxic or deterrent compounds expressed in floral nectar may perform a number of adaptive functions in plants, though are sometimes also associated with ecological costs, such as reduced pollinator visitation. The majority of previous investigations on toxic nectar stem from bee-pollinated species, however, and it is unknown whether toxic nectar is equally prevalent amongst other pollination syndrome types. Utilizing a diverse selection of *Rhododendron* species adapted to various pollinating guilds (i.e. bird, bee, moth, and butterfly), the primary aims of this study were to A.) assess the relative frequency as to which toxic nectar may be encountered amongst plants adapted to different pollinator types, and B.) examine patterns of trait evolution in both toxic nectar and pollination syndromes in the genus. Results indicated that co-evolution of specialized pollination syndromes has reduced the prevalence of toxic nectar amongst such species. Ancestral state reconstruction affirmed these patterns; in that loss of toxic nectar represents an evolutionarily advanced character in plants. Possible mechanisms behind these evolutionary patterns are discussed, which may be resultant, for instance, from rapid shifts between functional pollinator guilds, or as an additional barrier to nectar robbing in more open, generalized flowers which are otherwise not required in species which exhibit specialised floral morphology.

4.2 Introduction

Plant-pollinator coevolution is a continuous process which has been on-going since the first origins of entomophily around the Lower Cretaceous (Grimaldi 1999). A product of this co-evolutionary dynamic is observed in the form of pollination syndromes; suits of phenotypic traits that reflect convergent floral adaptation to one or several modes of animal pollination (be it bird, bee, moth, butterfly or other functional groups of pollen vector) (Fenster et al. 2004). While pollination syndromes as broadly defined may not always successfully predict the principle pollinator type for species (Ollerton et al. 2009), the central validity of the concept is none-the-less upheld on ecological and evolutionary grounds (Rosas-Guerrero et al. 2014). As a complement to morphological traits, floral nectar (on the basis of its energetic and nutritive composition; volume and rate of production etc.) commonly works in tandem in flowers as a co-adaptation geared towards attraction of specific pollinator groups (Nicolson and Thornburg 2007). The occurrence of so-called 'toxic' nectar, which is reported in many families (Baker 1977, Adler 2000), may likewise exist as a mechanism to filter floral interactions to a narrower range of more effective pollinators.

Although toxic nectar may also incur a cost to plants, depending on ecological context (Gegear et al. 2007), a number of adaptive functions to its expression have nonetheless been ascribed (Adler and Irwin 2005, Manson et al. 2010, Kessler et al. 2012, Wright et al. 2013). Perhaps key amongst these are enhancement of pollinator constancy (resultant in higher quality and/or quantity of pollen receipt), and protection from nectar robbers or non-adapted (and potentially inefficient) pollinators.

The majority of investigations to-date on toxic nectar stem from bee-pollinated species (Elliott et al. 2008, Koehler et al. 2012, Cook et al. 2013, Gosselin et al. 2013, Irwin et al. 2014, Tiedeken et al. 2014). This ecological bias towards bee-pollinated plants could be down to the wider prevalence of toxic nectar associated with this syndrome type (although this remains to be tested), but may also have been influenced by the fact that toxic nectar-producing plants hazardous to honeybees are just relatively more obvious to humans (Ott 1998). To a lesser extent, focus has also been afforded to toxic or deterrent nectar of bird-pollinated species (Struempf et al. 1999, Nicolson and Fleming 2003, Johnson et al. 2006, Lerch-Henning and Nicolson 2013); examples of which include the alkaloid-bearing nectar of *Nicotiana attenuata* and *Nicotiana glauca*, which is consumed by nectivorous hummingbirds and passerine sunbirds as their main pollinators, respectively (Tadmor-Melamed et al. 2004, Kessler et al. 2012, Ollerton et al. 2012). Much remains to be studied in relation to the occurrence and wider role of toxic or deterrent compounds in the nectar of bird-pollinated plants, although it is known, however, that even coevolved avian pollinators differ in their tolerance of nectar toxins, and may be deterred by high such levels (Nicolson and Fleming 2003, Kessler et al. 2012, Lerch-Henning and Nicolson 2013). Why nectar toxins are maintained in such species may however relate to the fact birds can also act as nectar-robbers (Kaczorowski et al. 2014). Outside of these pollinator groups, few studies to-date have focused on Lepidoptera as major pollinators of toxic nectar-containing plants (Landolt and Lenczewski 1993, Adler and Bronstein 2004, Adler et al. 2006).

A comparative study conducted across *Nicotiana* revealed that alkaloid levels in nectar negatively correlate with the extent to which species rely on out-crossed pollination (via entomophily) to achieve reproductive fitness (Adler et al. 2012). The need for such compromises, together with what can be variable selective pressures from pollinators (Chapter 3), reveal the challenge presented to plants in balancing expression of toxic nectar in relation to mutualists' needs. In this regard, pollinator-mediated selection, if sustained over sufficient time, may also conceivably act to eliminate expression of toxic nectar in species altogether (Chapter 3). Beyond these indications, however, little to no attention has been afforded to the topic of toxic nectar and pollination syndromes generally. In particular, the relative frequency as to which toxic nectar may be encountered amongst plants adapted to different pollinator types remains unknown, alongside historic patterns of evolution in both these traits.

We here examined this topic within the genus *Rhododendron* L. (Ericaceae) – which due to the suspected widespread occurrence of toxic nectar amongst species (from preliminary evidence), and the large diversity of pollination syndromes within the genus (Fig. 1.); formed an ideal model in which to investigate these and related questions. We focused in particular on species of sections Pontica, Rhododendron and Vireya. These taxa exhibit a wide gradient in terms of pollination syndromes, ranging from more generalized, bee-pollinated plants in section Pontica, towards increasingly specialised butterfly, moth and bird-pollinated species in some section Rhododendron species and virtually all Vireya (Stevens 1976, Stevens 1985). While the former sections mostly exhibit a Eurasian distribution, species of section Vireya (colloquially known as tropical rhododendrons) in contrast hold a centre of endemism within the Malesian floristic region (Argent 2006). Evidence supports the hypothesis that recent evolutionary divergence between sections Rhododendron and Vireya was perpetuated by a rapid adaptive radiation in the latter, following a linear progression along the Malesian Archipelago (Brown et al. 2006b). A shift in Vireya from bee or mixed pollination systems which elsewhere typify the genus (Mejías et al. 2002, Escaravage and Wagner 2004, Stout et al. 2006, Stout 2007a, Ono et al. 2008, Kudo et al. 2011), to bird, moth, and butterfly (as well as possibly bat) pollination syndromes, is thought to have functioned as a key innovation in this radiation (Stevens 1976, Stevens 1985).

Our aims in this study were therefore to A.) examine taxonomic and evolutionary patterns in pollination syndromes and the distribution of diterpene secondary metabolites (known as grayanotoxins; GTXs) in nectar across taxa of the genus *Rhododendron*; and B.) assess whether the presence/absence of nectar GTXs and other functional attributes (such as plant height) are predictive of pollination syndromes in *Rhododendron*, which may therefore permit greater insight into their historic and present evolution.

4.3 Methods

4.3.1 Focal taxa

A broad range of species exhibiting diversity in pollination syndromes were selected for inclusion in this study. We targeted species of sections Vireya and Rhododendron (subgenus *Rhododendron*) and section Pontica (subgenus *Hymenanthes*) because of their diversity and contrast in pollination syndromes, as well as their wide representation in cultivated collections held at the Royal Botanic Gardens Edinburgh (UK) and the National Botanic Gardens, Glasnevin (Ireland), from which nectar could be sampled (see below). We assigned all species which were sampled into taxonomic groupings. As a whole, 114 species were sampled across three subgenera, six sections, and 35 subsectional groupings (Appendix 4). For sections Pontica, Rhododendron and other minorly represented taxa, infrageneric classification followed that of Chamberlain et al. (1996) as modified by Goetsch et al. (2005). In place of subsections for section Vireya, we followed clade-based

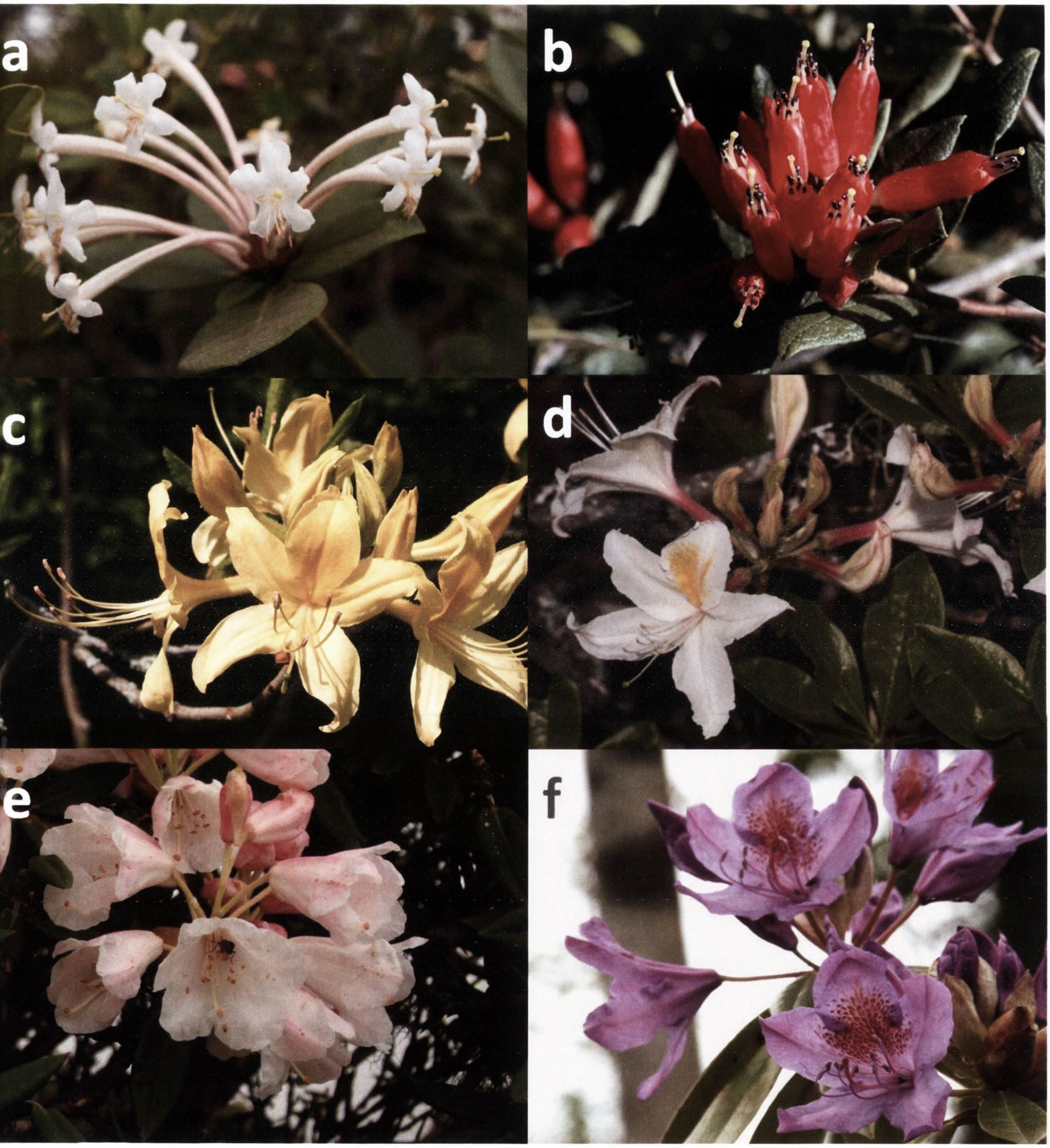

Figure 1. Representative gradient of pollination syndromes found in *Rhododendron*, from top to bottom in order of increasing syndrome generality, from moth and bird pollinated species, to butterfly and bee / generalist pollination. Depicted species, and their respective taxonomic sections, include: (a.) *R. herzogii* Warb. – Vireya; (b.) *R. spinuliferum* Franch. – Rhododendron; (c.) *R. luteum* Sweet – Pentanthera; (d.) *R. occidentale* (Torr. & A.Gray) A.Gray – Pentanthera; (e.) *R. pseudochrysanthum* Hayata – Pontica; (f.) *R. ponticum* L. – Pontica. Credit: Wikimedia Commons (photos a-e), P. Egan (photo f).

groupings reported by Brown et al. (2006a) – which due to their high biogeographical accordance allowed us to tentatively assign sampled species into evolutionarily-relevant groups, despite the fact these several have not been included in phylogenetic analyses to-date. The sectional name 'Vireya' is used here informally throughout, as the predating name of 'Schistanthe' is now technically considered correct (Craven et al. 2011).

4.3.2 Nectar sampling and analysis of GTXs

Nectar was sampled from flowers using either 1 or 5 µl glass microcapillary tubes, depending on the volume of nectar present in flowers. 5-30 µl of nectar was usually obtained per species and pooled into a single sample per individual plant. Replicate samples were taken to the best extent possible, as permitted by the number of individuals cultivated and in flower per species (usually between one and six). Nectar samples were freeze-dried in the lab, to remove water content, and subsequently re-suspended in 200 µl MeOH for analysis. Nectar of all 113 sampled species was screened for the presence/absence of GTXs. LC-MS analysis for detection of GTXs was conducted as previously reported (Chapter 2). While about 28 grayanane-type diterpenoids are known from *Rhododendron*, systematic reviews report that GTX I is the most widely occurring (or at least most commonly reported) GTX in the genus (Qiang et al. 2011, Li et al. 2013). We thus employed GTX I, as well as GTX III, as a marker for the presence of GTXs in nectar generally – though in addition recorded any other GTXs positively detected in extracted ion chromatograms of the *m/z* 411.1 and *m/z* 415.3 molecular ions, which are diagnostic of many GTXs. The reliability of these compounds as joint markers of the general presence of GTXs in nectar was evidenced by the fact that in only 3.4 % of cases were other GTXs recorded in their absence. We none-the-less considered that false negatives were still possible. However, low nectar volume was at least ruled out as a source of these, as we established through logistic regression that sample volume was not a significant predictor of the presence/absence of nectar GTX (data not shown).

4.3.3 Floral traits and categorization of pollination syndromes

Floral morphological traits selected for the characterization of pollination syndromes are presented in Table 1, alongside their unit/scale of measurement. Data on these floral traits, as well as other ecologically relevant attributes (including plant height and elevation of occurrence), were assembled from across several standard monographs and other taxonomic sources covering the genus *Rhododendron* (Cullen 1980, Chamberlain 1982, Fang et al. 2005, Argent 2006, Mao and Gogoi 2012). While usually a large variety of floral traits are important in defining pollination syndromes, our selection of which to include in our analyses was limited to those consistently reported across the literature for all species. Hence, more finer and potentially important characters were not included due to a paucity of documentation, such as the angle of flower orientation (erect, pendant etc.), the degree of excision and curvature of styles and stamens, and stamen arrangement within flowers (whether circular or zygomorphic). Considerable infraspecifc diversity in some traits (such as flower colour or corolla shape) exists for a number of species. In these circumstances we took the predominant state mentioned, and/or the character state present on the individual from which nectar was sampled, where these were obvious to us. Floral traits were used to differentiate species pollination syndromes through an unsupervised ordination (see below). However, we also assigned *a priori* pollination syndrome categories to species based on criteria described by Stevens (1976) for moth, bird and butterfly pollinated flowers. Although originally employed for Malesian *Rhododendron*, we considered valid the wider use of this system within the genus. Species which did not satisfy any of these criteria were assigned a 'general' pollination syndrome.

Table 1. Floral morphological traits and attributes recorded per species

Trait	Scale	Character states / unit of measurement
Floral traits		
Pedicel length	continuous	cm
Corolla colour red*	continuous	PCA axis 1
Corolla colour green/blue*	continuous	PCA axis 2
Corolla base broadness†	ordinal	1 = tubular; 2 = funnel; 3 = campanulate; 4 = saucer/rotate
Corolla mouth broadness†	ordinal	1 = tubular; 4 = campanulate; 5 = funnel; 6 = saucer/rotate
Corolla length	continuous	cm
Corolla tube length	ordinal	0 = absent; 1-3 = in 20 mm intervals; 4 = 61-80 mm +
Corolla tube curvature	binary	0 = actinomorphic; 1 = zygomorphic
Scent^	ordinal	0 = absent; 1 = fragrant; 2 = strongly fragrant
Nectar guide or blotches	ordinal	0 = absent; 1 = few or faint; 2 = extensive
Other attributes		
Plant height	continuous	m
Mean elevation	continuous	m

* Colour was quantified on a continuous scale through PCA analysis (Yoshioka et al. 2004), based on unique combinations of red, green and blue (RGB) values (Appendix 5). As potentially more relevant ecologically, we prioritised the colour of lobes over the colour of tubes, where these differed

† The difference between corolla types in angle of divergence at the base/mouth follows a general scheme for classification of *Rhododendron* flowers, as depicted in Davidian (1992). Scores for corolla mouth broadness were off-set to account for mixed flower types (i.e. those which possessed one corolla type at the base grading into another towards the opening; e.g. tubular-funnel, funnel-campanulate etc.). Consequently, all corolla types possessed a unique score combination, while maintaining an ordinal scale.

^ We consider this trait to be significantly under-reported in the literature, and treat its use with caution

4.3.4 Data analysis

Taxonomic distribution of nectar GTXs – Differences in the proportion of species with nectar GTXs between sections Rhododendron, Vireya and Pontica was assessed through a generalized linear mixed model (GLMMs) fit by maximum likelihood in the R package lme4 (Bates et al. 2013). For this, we summarized the proportion of species with and without detected nectar GTX at the subsection level, as the unit of replication within sections (mean no. subsections per section = 7; species per subsection= 4). A binomial family was specified, as suited for analysis of proportional data, with 'section' included as a fixed effect, and 'subsection' included as a nested random effect. We excluded from the analysis subsections which contained only a single representative species. However, leaving these subsections in did not alter the overall results (data not shown). Post-hoc Tukey pairwise comparisons were used to determine which sections differed significantly from one another, using the R package multcomp (Hothorn et al. 2008), and corrected for multiple comparisons by Benjamini-Hochberg adjustment. We assessed the GLMM for possible over-dispersion, and further validated the model through evaluation of standardized residuals for non-normality and homogeneity, as well as assessment against a null model.

Assessment of pollination syndromes – Ordination of species based on their floral traits within pollination syndrome 'space' was made through non-metric multidimensional scaling (NMDS); an unconstrained ordination technique suited to ordinal data. NMDS was conducted in the R package vegan (Oksanen et al. 2013) in two dimensions, employing a Jaccard dissimilarity index, and set for a maximum of 1000 iterations for convergence. As the range of values in the dataset was large, a Wisconsin standardization was used, which can often help to improve gradient detection ability (Oksanen 2013). In order not to artificially inflate differences between species, and to produce more ecologically interpretable ordinations, we limited the selection of variables included in the NMDS to floral traits which, according to Spearman rank correlations, were not more than moderately correlated which each other (i.e. Spearman's rho ≤ 0.45). Selection of the best sub-set of traits to include was achieved by fitting a small number of alternative ordinations, and selecting that which best fit the data according to the stress goodness-of-fit statistic. Stress of the final ordination was low ($S = 0.1037$), indicating a high goodness of fit (Clarke 1993).

Relation of pollination syndrome generality to species attributes and nectar GTXs – Species scores from Axis 1 of the NMDS ordination were further employed in subsequent analyses, as this axis was observed to represent a strong gradient between specialist and more general types of pollination syndrome (see results). To examine whether plant height and species' mean elevation of occurrence were significantly associated with the generality of pollination syndromes (i.e. Axis 1 scores), we fitted linear mixed models (LMMs) using the package nlme (Pinheiro et al. 2013). We specified the continuous variables of plant height and mean elevation as fixed effects in the LMM, together with the categorical factors of subgenera, and section nested within subgenera, as random effects. This particular model structure therefore allowed us to assess if any such patterns hold universally, regardless of the particular taxonomic section considered. Model validation was carried out the same as for the GLMM above. In addition, model residuals were assessed for phylogenetic signal (see below), in order to ensure the statistical assumption of non-independence was not violated (Kamilar and Cooper 2013).

To examine if the binary presence or absence of nectar GTX in species could be predicted by the generality of pollination syndromes, we fit a generalized linear model (GLM) with a binomial error structure. Unlike binomial models which consider proportional data, model overdispersion is not possible with a binary response variable, and so the GLM goodness-of-fit was assessed in comparison to a null model. Similar as for the above LMM, phylogenetic signal in model residuals was checked.

Ancestral state reconstruction and phylogenetic signal – A maximum likelihood phylogram based upon molecular sequencing of the *RPB2* nuclear gene by Goetsch et al. (Goetsch et al. 2005) was accessed from TreeBase (Tr2071) and utilized in this study. As trait information assembled in present study was only available for 23 species in the phylogram, we pruned the tree to match this set of taxa. Ancestral state reconstruction for characters was performed under both maximum parsimony (MP) and maximum likelihood (ML) in Mesquite version 3.01 (Madison & Madison, 2014). Presence/absence of nectar GTXs (as a binary trait), and pollination syndromes generality (as a continuous trait) were mapped onto this typology, and treated as unordered characters, in which any state change is counted as one step. As ML ancestral reconstruction is currently only supported for categorical data (Madison & Madison, 2014), this was only performed in addition for nectar GTX. For this, an Mk1 model was applied, in which any change between states is considered equally probable. Phylogenetic signal in each character was tested in the R Caper package (Orme 2013), utilizing phylogenetic generalised least squares (PGLS) to provide maximum likelihood measurement of λ (Pagel's lambda) for continuous traits, and the 'phylo.d' function for calculation of the *D* statistic; a measure of phylogenetic signal for binary traits (Fritz and Purvis 2010). Additionally, the residuals from the above conducted LMM and GLM models were assessed for phylogenetic signal. In both cases, λ did not significantly differ from zero (i.e. exhibited no phylogenetic signal), thus justifying the use of standard non-phylogenetic models (Losos 2011).

4.4 Results

4.4.1 Taxonomic distribution of nectar GTXs

Out of 113 species of *Rhododendron*, nectar GTXs were detected in 34 species (38.4 %). The taxonomic pattern of occurrence of nectar GTXs was not random, however (Fig. 2): Vireya was found to be devoid of nectar toxins, whilst sections Pontica and Rhododendron had significantly more species with nectar GTXs (binomial GLMM: $F_{2,21}$= 4.1, p= 0.006).

4.4.2 Assessment of pollination syndromes

A multivariate analysis of floral morphological traits was successful in differentiating functional groupings of pollination syndromes amongst taxa (Fig. 3). Axis 2 of the NMDS ordination mostly separated out species within sections and pollination syndrome types based on corolla colour and pedicel length, whereas Axis 1 separated out species between sections and syndrome types based on tube length and broadness of the corolla base. This large gradient evident along Axis 1 ranged from species with specialised pollination syndromes to those showing increasing generality. We thus subsequently took these Axis 1 scores as a continuous measure of the generality of pollination syndromes in species. The legitimacy of such was further supported by the finding that our *a priori* categorized pollination syndrome types (as shown in Figure 3) were significantly differentiated in their Axis 1 scores (ANOVA: $F_{3,99}$= 58.1, p= ≤0.001, R^2= 0.63), with species that exhibited bird, butterfly and moth pollination syndrome types each differing significantly in Axis 1 scores to those with a more generalized pollination syndrome (Tukey pairwise contrasts significant at $p \leq 0.05$).

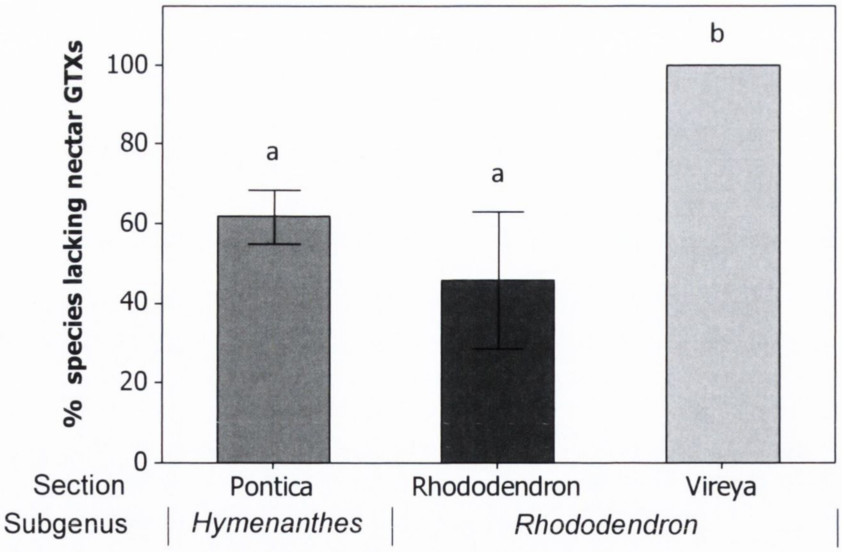

Figure 2. Taxonomic distribution of nectar GTXs within target taxa of *Rhododendron*. Means (± SE) which do not share a letter differ significantly (at $p \leq 0.05$) according to Tukey pairwise contrasts (corrected for multiple comparisons). Comparisons were made based on the proportion of species per section with or without nectar GTXs, with subsection included as a nested random effect. Subsections with only a single representitive species were precluded from the analysis (see methods).

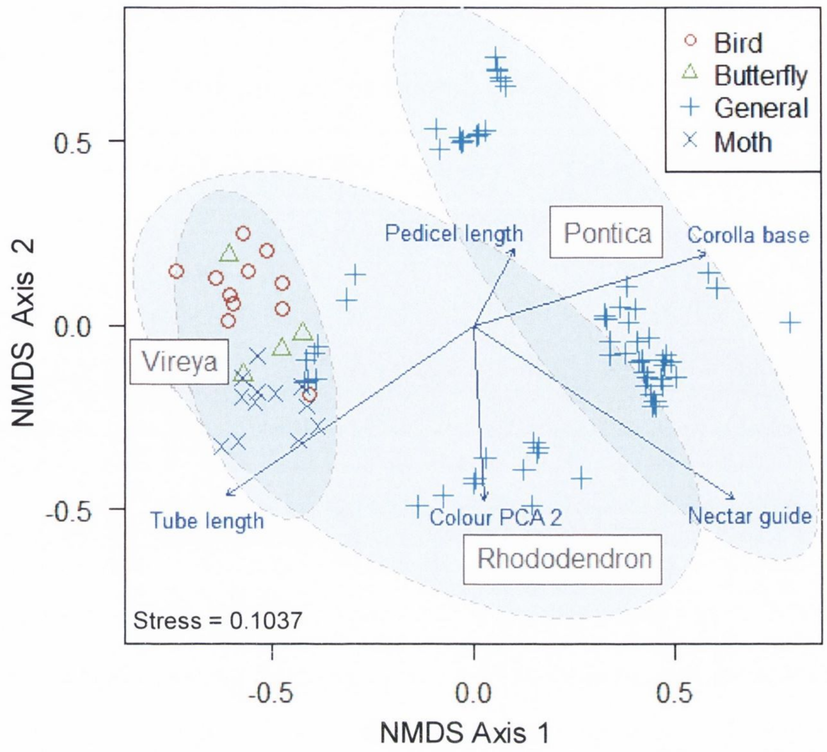

Figure 3. Multivariate ordination of pollination syndromes through non-metric multidimensional scaling (NMDS). The contribution of floral traits to ordination axes is indicated by the direction and relative length of vector arrows (see Table 1 for labels). Taxonomic sections are bounded by 95% confidence interval ellipses (based on SE), and are significantly differentiated in pollination syndrome 'space' (permutational MANOVA; $F_{2,102}$= 13.1, p= ≤0.001, R^2= 0.21).

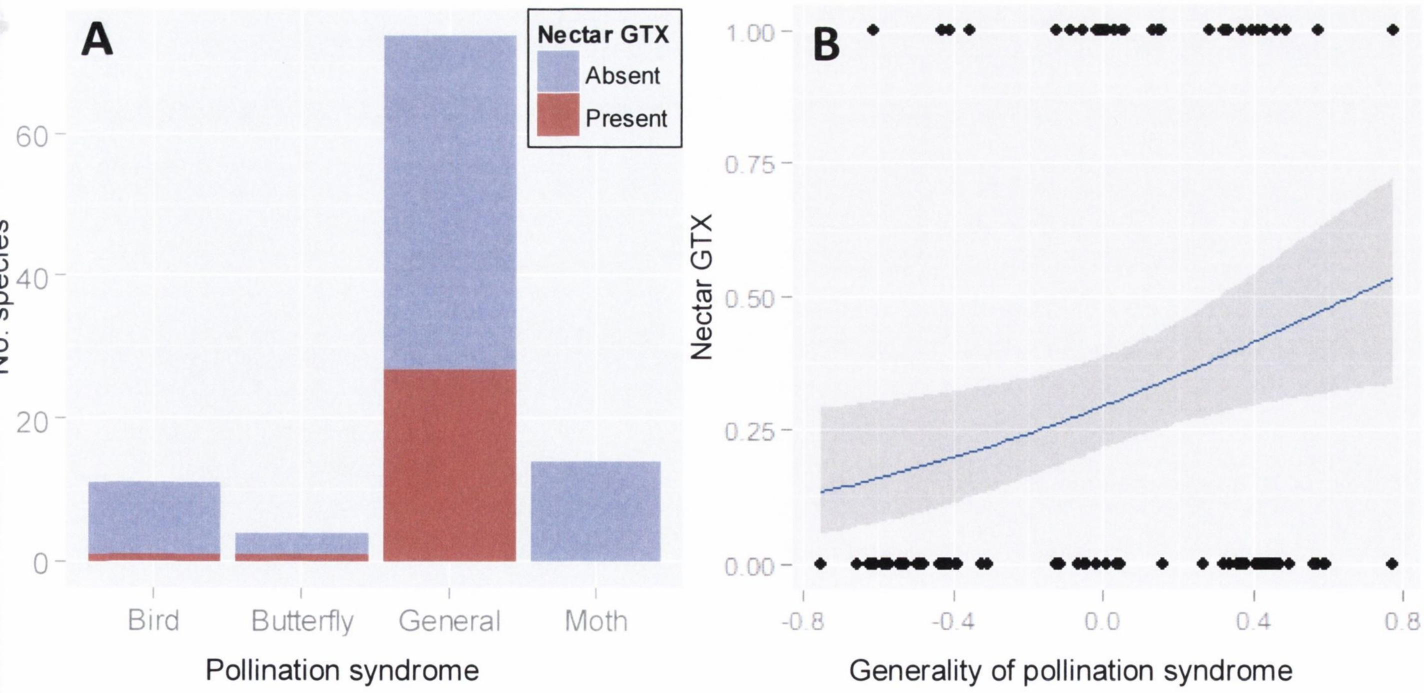

Figure 4. (A.) Frequency of species exhibiting nectar GTXs by pollination syndrome category. The 'general' category refers to species which possess floral traits mostly apparently suited to bee-pollination, but which may also be visited by several other pollinator types. (B.) Binary regression of generality of

pollination syndromes (based on NMDS Axis 1 – ranging on a scale from highly specialised towards increasing generalization) against presence/absence of nectar GTXs. The predicted probability (± SE) of nectar GTXs across species rises as pollination syndromes become more general.

In relating species' level of generality of pollination syndrome to other ecologically important attributes, we detected a significant association between pollination syndrome generality and plant height, though not for species' elevation of occurrence (Table 2.). Given the specific nested structure fitted in this LMM, these results therefore indicate that taller species tend to possess less specialised pollination syndromes, and that this pattern holds across taxonomic sections (e.g. that taller species of Vireya tend to be relatively less specialised than smaller species within this section etc.).

4.4.3 Evolution of pollination syndrome generality and nectar GTXs

In contrast to more generalist pollinated species, in which nectar GTXs were relatively common, the prevalence of nectar GTX amongst bird, butterfly and moth pollinated species was observed as very low (Fig. 4a). The level of generality of pollination syndromes in species could successfully account for the likelihood of occurrence of nectar GTXs (binomial GLM: deviance = 114.9, *df*= 101, *p*= 0.006), indicating that the probability of nectar GTXs increases as pollination syndromes become more generalized (Fig. 4b).

Based on ancestral state reconstruction, toxic nectar was found to represent the ancestral state in *Rhododendron* (Fig. 5). Both parsimony and ML reconstructions were in accordance with this finding. An intermediate level of pollination syndrome generality is supported as the ancestral state, from which diversification into more specialised and generalised pollination syndromes has most likely occurred. In tracing the history of character evolution on the tree, significant phylogenetic signal was detected for toxic nectar (D = 0.048), with low probability this estimate has resulted from random phylogenetic structure (p= 0.035). Similarly, strong phylogenetic signal was detected in pollination syndrome generality (λ = 1), allowing rejection of the null hypothesis that lambda is equal to zero (p= 0.003).

Table 2. Linear mixed model (LMM) of the relationship between the response of pollination syndrome generality and plant height. Subgenera, and sections nested within subgenera, were fit as random effects; $N_{[\text{subgenera}]}$ = 3, $N_{[\text{sections}]}$ = 6, $N_{[\text{species}]}$ = 114.

Model$^{\#}$	**Coefficient** (± SE)	***t*-value**	***p*-value**
Fixed effects			
Intercept	-0.064 (± 0.172)	-0.37	0.710
Plant height	0.012 (± 0.005)	2.22	**0.029**
Random effects	Variance Component		
subgenus	0.060	-	-
subgenus/section	0.043	-	-
residual	0.050	-	-

$^{\#}$A likelihood-ratio test indicated that mixed model AIC was significantly lower than a null model containing random effects only (L = 4.92, df= 1, p= 0.027)

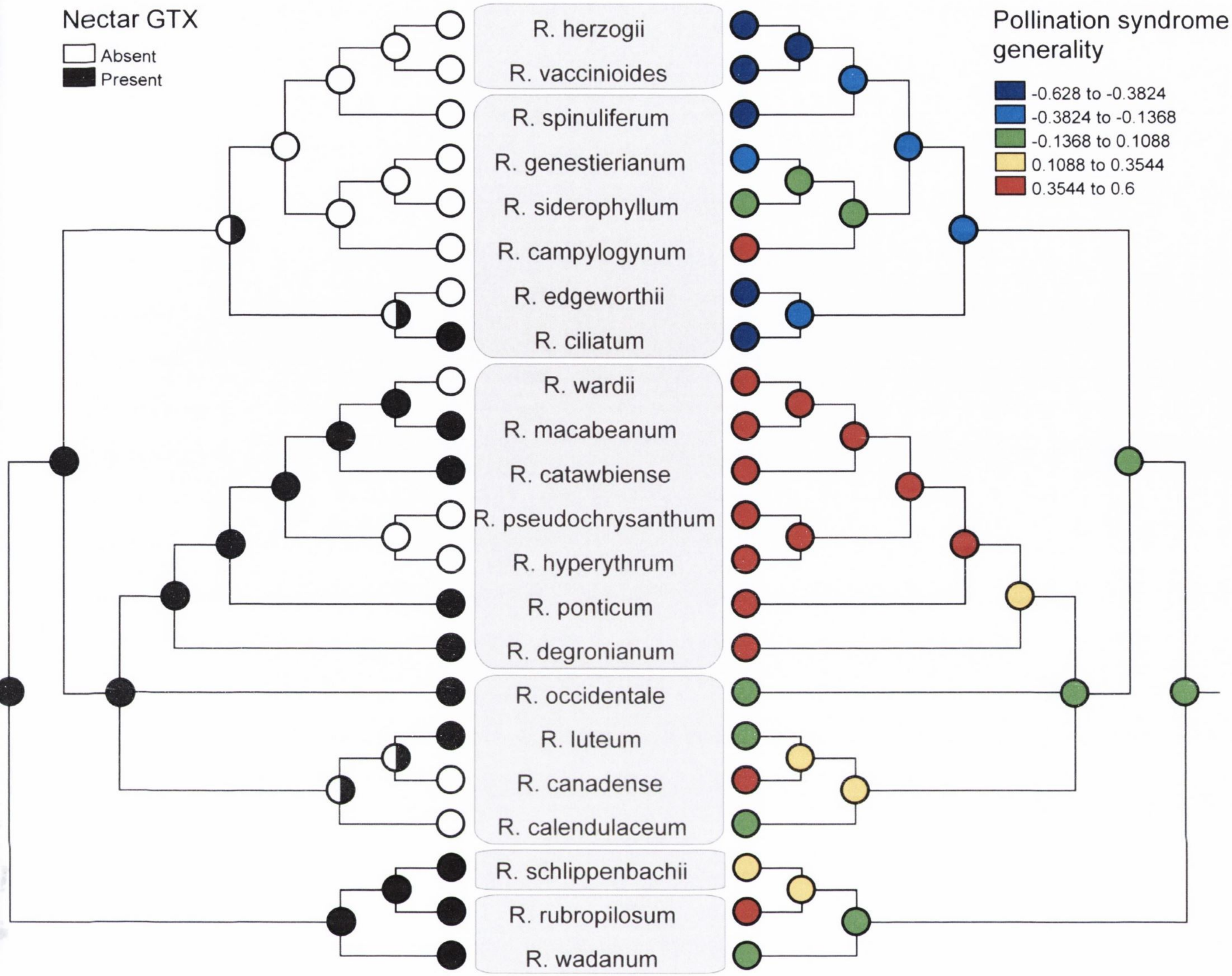

Figure 5. Ancestral state reconstruction of toxic nectar (left) and pollination syndrome generality (right). Parsimony reconstruction is depicted for both characters. Grey boxes designate taxonomic sections (which from top to bottom include: Vireya, Rhododendron, Pontica, Pentanthera, Sciadorhodion and Tsutsusi).

4.5 Discussion

We documented the occurrence of pollination syndromes within several taxa of *Rhododendron*; which were assigned firstly on the basis of multivariate ordination of floral traits, and secondly through *a priori* categorization (according to established criteria). In doing so, we revealed that: A.) there is an evolutionary non-random distribution of nectar GTXs amongst taxa; B.) there is a tendency for species with smaller plant height to possess more specialised pollination syndromes; and C.) pollination syndrome is predicative of the occurrence of nectar GTX.

Accordingly, we demonstrate for the first time that presence of toxic nectar varies amongst species according to pollination syndrome type, and that loss of toxic nectar can be considered a more derived trait in clades. These findings also corroborate the apparent literature bias towards toxic nectar of bee-pollinated species; as a comparatively higher prevalence of toxic nectar amongst species of this syndrome type in the *Rhododendron* genus was affirmed, relative to other pollination syndrome types. However, while our detection of GTXs in species' nectar may to some extent have been subject to false negatives (see methods), we at least expect these to have been taxonomically random, and therefore not of large consequence to overall patterns.

The distinct patterns of occurrence of toxic nectar across pollination syndrome types, as here observed, begs the question as to why (and through what potential mechanisms) these patterns have evolved. Phylogenetic studies on the genus point to a relatively recent split between species of sections Vireya and Rhododendron (Brown et al. 2006a, Craven et al. 2011). Given the relatively common occurrence of nectar GTXs within section Rhododendron, together with a generalized pollination syndrome (albeit specialisation is observed in some species); our findings suggest that evolutionary divergence in section Vireya towards more specialised forms of pollination has been accompanied by loss of toxic nectar. While a reasonable tolerance to GTXs is known in some invertebrates, such as for species of bee and specialist geometrid moths (Nishida 1995, Tiedeken et al. 2014); vertebrates are more widely prone to adverse effects from GTX ingestion, via a neurotoxic mode of action (Jansen et al. 2012, Popescu and Kopp 2013). Anecdotal evidence in addition suggests the avian toxicity of GTX I (Marsh and Clawson 1930, Bloom and Grivetti 2001). Shifts from bee to bird, and bird to moth/bat pollination syndromes (which may also have occurred in Vireya) represent relatively common transition paths in the evolutionary course of angiosperm taxa (Rosas-Guerrero et al. 2014). As here supported by ancestral state reconstructions, loss of toxic nectar may therefore have formed an integral adaptation in Vireya, underpinning a shift from bee to bird and other specialised forms of pollination. Our past findings in *R. ponticum* indeed demonstrate how selective pressures exerted by pollinators may bring about reduced expression of nectar GTXs (or even their complete loss) within newly colonized ranges, as a functional adaptation to novel mutualist assemblages (Chapter 3).

That species exhibiting more generalized pollination syndromes were more likely to contain nectar toxins can also be looked at from the functional perspective of chemical defence. It may be reasoned that flowers which are more accessible in a morphological sense may require additional barriers in order to defend against nectar robbing. Whereas in contrast, this is less of a requirement in more specialised flowers, in which access to nectar is restricted by a more elaborate morphology co-evolved with specific pollinators. However, explanations as to why flowers lack expression of toxic nectar need not be mutually exclusive. This could be due both to lack of a need to chemically defend nectar (because of floral specialisation and/or the absence of antagonistic selective pressures), or the need to minimize negative impacts on legitimate pollinators. Hence, in addition to specialized species, those exhibiting generalized pollination syndromes may also none-the-less lack nectar GTXs (e.g. as in certain plants/populations of *R. ponticum* – Chapter 2). Likewise, a small number of bird and butterfly-pollinated species were found to contain nectar GTXs (e.g. *R. keysii* Nutt., *R. cinnabarinum* Hook.f.) – which presumably may serve a defensive function, but do not impact on pollinators.

4.5.1 Conclusions

As a whole, this study contributed novel findings on the taxonomic distribution of toxic nectar, and how such patterns may be explained in an ecological and evolutionary context. Similar to other wide taxonomic assessments of toxic nectar-containing species (Adler et al. 2012, Manson et al. 2012), such insights were only possible due to the large comparative approach undertaken. That pollination syndromes were found to as sufficiently predictive of the likelihood of encountering toxic nectar in species therefore represents a significant step forward in our understanding of the evolutionary causes and consequences toxic nectar. Future studies which utilise a taxonomically broad sampling approach may therefore permit further progress.

Chapter 5

Invasive alien species threatened with native extinction – putting conservation translocation of climate-endangered species to the test

"Real-world biogeographers must balance their roles as citizens wanting to make the world a better place against their roles as scientists who are honest and skilful about their scientific limitations. Getting the science right is undoubtedly important for making policy decisions, but a wise approach to uncertainty is all the more so."

– Koy Thomson

5 Invasive alien species threatened with native extinction – putting conservation translocation of climate-endangered species to the test

5.1 Abstract

Assisted migration is generally considered the last line of action for averting species extinction. While the theoretical foundations which underpin this conservation strategy have steadily progressed over the last two decades; deliberation within the specific context of climate change remains in urgent need of address. The juxtaposition of invasive alien species threatened with extinction in their native range could prove useful to demonstrate both the potential risks and benefits associated with translocation as a conservation tool. I here build a case-study on the Iberian tertiary relict *Rhododendron ponticum* ssp. *baeticum* (Ericaceae), which in its native range is subject to threats common to many climate-endangered taxa. I demonstrate use of a decision framework designed for the identification of temporally stable climate change microrefugia potentially suited for translocation, and which minimize invasion risk through prioritizing the selection of candidate sites based on geographic proximity to the extant range.

Simulations of future migration for *R.p.* ssp. *baeticum* under several climate change scenarios were initiated utilizing high-resolution species distribution models (SDMs) coupled to a population spread model. It was thus revealed that climatic suitability for this species within its extant range is set to rapidly decline. Under the most pessimistic scenario of climate change, by 2050, it was predicted that dispersal limitations will have prevented the species from occupying any area of suitable climate. The framework developed as part of the current study was successful in identifying candidate sites potentially suited for conservation translocation by the end of the current climate period (i.e. 2020). Explanation as to why SDMs performed well according to model evaluation, yet did not predict suitable climate in the invaded range, may relate to the high specificity of *R.p.* ssp. *baeticum* to relict habitat in the native range. While on one hand such hidden plasticity may lead to failure in predicating invasive capacity in novel geographic areas (for this species and other narrow endemics generally), this discrepancy would also lead to more conservative estimates of areas suitable for translocation, which therefore minimize risk of invasive spread.

5.2 Introduction

One of the few viable solutions for averting species extinctions under rapid climate change may be the intentional introduction, or translocation, of highly threatened species between geographic regions (Hunter 2007, Ledig et al. 2012). However, considerable controversy surrounds the selection of geographic areas for such purposes (Ricciardi and Simberloff 2009b, IUCN/SSC 2013). If a species is translocated to an area within its historical distribution, this is referred to as 'reintroduction', but if moved to a novel location, this is referred to as 'assisted colonization' (Seddon 2010). Straightforward cases therefore involve reintroduction of species into areas of recent former occupancy, where local extinction has been directly attributed to anthropogenic influence (Baker et al. 2011, Nussear et al. 2012). However, translocation outside of a species' historical range may offer the only realistic prospect for curtailing extinctions in more complex cases (Thomas 2011), such as where A.) a historical range is unknown, B.) there is little or no suitable habitat within the historical range (either presently or projected with climate change), and C.) sites for suitable translocation are not within close (bio)geographic proximity to the extant range.

Although translocation outside of a species' historical range has been justified accordingly, on hypothetical grounds, few practical examples have emerged so far, limiting insight into the technical processes and risks associated with this conservation strategy (McLachlan et al. 2007). In particular, significant challenges are presented in the form of identification of recipient sites suited for translocation, and risk assessment of negative impacts such as potential aggressive invasion (Mueller and Hellmann 2008). As such, translocations are generally considered the last line of action for averting species extinction, due in part to these relatively high cost and risk factors (Shoo et al. 2013). While the intentional movement of species at inter-continental scales has given rise to many invasive species and serious unintended impacts globally (Dehnen-Schmutz et al. 2007, Ricciardi and Simberloff 2009a), such types of translocation are not advocated under assisted colonization for conservation purposes (Thomas 2011). While the theoretical foundations which underpin this conservation strategy, and in particular assisted colonization, have steadily progressed and refined over the last two decades; deliberation within the specific context of climate change remains in urgent need of address. Recent elaboration of the concept of climate change refugia (Ashcroft 2010, Keppel et al. 2012), has provided a theoretical basis for the identification of species-specific safe havens under future changing climate. Depending on the dynamics of such safe havens, these may be termed as microrefugia, holdouts or stepping-stones that facilitate species range shifts (Hannah et al. 2014). Despite the potential to better guide translocation practise, however, specific frameworks to support the selection of climate-stable habitat along these lines remain in need of development.

As a means to gain insight into some of these issues, we here collate examples of a number of invasive alien species (IAS) which, paradoxically, are threatened with extinction in their native range due to climatic and/or anthropogenic factors (for examples of such species see Appendix 6). Given this juxtaposition, these species may therefore be useful to demonstrate both the potential risks and benefits associated with translocation as a conservation tool. In particular, we build a case-study on a well-known plant species: the Iberian tertiary relict *R. ponticum* ssp. *baeticum* (Ericaceae). Although highly invasive in NW Europe (Cross 1975b, Mejías et al. 2007), within its native range this species is subject to threats common to many climate-endangered taxa (Reinhardt et al. 2005, Early and Sax 2011, Thomas 2011), in that potential range shifts are constrained by: (i) high habitat specificity (within regionally rare or relict habitats); (ii) an inherent low dispersal ability; and (iii) the likely formation of insurmountable climatic barriers in the short to medium-term. We utilize spatially-explicit spread models (integrating projections of shifts in climatic suitability devised from species distribution models, with dynamic simulations of demography and seed dispersal), to forecast the likelihood for migration and survival of *R. ponticum* in its native range under climate change. Species distribution models (SDMs) are a standard recommended tool for purpose of informing translocation (IUCN/SSC 2013), and in particular, offer promising prospect for identification of putative microrefugia under future climate conditions (Ashcroft 2010, McLaughlin and Zavaleta 2012). Yet their use for such inference has been limited to-date (Keppel et al. 2012), and not explicit to conservation translocation. The specific limitations of SDMs have been well-noted (Guisan and Thuiller 2005), and it is recognised that uncertainty associated with differences between SDM model techniques (i.e. statistical uncertainty), as well as future climate change scenarios (i.e. climatic uncertainty), can be considerable (Franklin et al. 2014). While development of ensemble forecasting has helped address the challenge of statistical uncertainty (Araújo and New 2007, Thuiller et al. 2013), ensemble predictions themselves can also vary due to the various model averaging methods employed.

As a practical output from this case-study, we demonstrate use of a decision framework designed for identification of temporally stable climate change microrefugia which are potentially suited for translocation. This framework explicitly seeks to minimize invasion risk through prioritizing the selection of candidate translocation sites based on geographic proximity to the extant range, rather than use of a strict biogeographic criterion – as such delimitations and their usefulness are specific to present day climate (Williams et al. 2007, Mahlstein et al. 2013), and hence diminish for predictions based on future climate scenarios.

5.3 Methods

5.3.1 *Climate change data and scenarios*

Baseline and future climate change datasets developed by the EcoChange Project (Dendoncker et al. 2008) were used to undertake species distribution modelling at a 2 km resolution. This climate dataset was derived from Worldclim data (Hijmans et al. 2005) downscaled to a finer 100 m. Downscaling was achieved through process of i) determining the relationships between climate and various topographical measures at the regional scale, ii) scaling the information determined in step 1 down to a finer resolution using spatial interpolation; and iii) combining the downscaled information with existing higher resolution DEMs to re-generate the climate grids at this finer resolution. For the current study the EcoChange finer resolution raster data were aggregated to 2 km resolution using bilinear interpolation implemented in R. From an originally more extensive set of environmental variables, six variables were used to undertake the modelling, including three temperature variables, two precipitation variables, and potential evapotranspiration (Appendix 8). This sub-set of variables was selected based on their demonstrated performance in models, but also on their readily interpretable ecophysiological link to this species' fundamental niche (Cross 1975b, Shaw 1984, Griffin 1994, Mejías et al. 2002, Mejías et al. 2007, Harris et al. 2009). Our projections of future climate change impacts were based on the ECHAM5 general circulation model (GCM), which was coupled to the RCA30 regional climate model (Jones et al. 2004). Predictions were generated for two time slices (2021-2050 and 2051-2080), and three different IPCC emissions scenarios (A1B, A2, and B2). These emissions scenarios reflect different assumptions about demographic, socioeconomic and technological development on greenhouse gas emission (van Vuuren et al. 2010).

5.3.2 Species distribution modelling

A presence-absence dataset for *R. ponticum* ssp. *baeticum* was assembled from field surveys conducted throughout 2011 and 2012 across the disjunct regions of the species' native Iberian range (in southern Spain, and southern and northern Portugal). Spatial coordinates were recorded to high accuracy with a handheld GPS (Garmin Colorado 300). Field records were then combined with spatial coordinates obtained from literature sources (Erfmeier and Bruelheide 2004, Perez Latorre and Cabezudo 2006, Mejías et al. 2007), to give a final presence-absence dataset. This final dataset consisted of almost 200 unique occurrence points that were mostly non-aggregated in space (i.e. ≥ 50 m distance), and of resolution greater that the 2 km extent used for climate data.

Current and future projected climatic suitability for *R. ponticum* ssp. *baeticum* was predicted using an ensemble of four modelling techniques (i.e. Random Forests, Generalised Boosting Models, Generalised Additive Models, and Maxent) available in the BIOMOD2 library (Thuiller et al. 2013), and implemented within the R statistical programming environment (R Development Core Team, 2013). We used a range of ensemble model (EM) algorithms to combine the outputs from the four SDMs, including mean of probabilities; median of probabilities; committee averaging; and weighted mean of probabilities. Pseudo-absences (10 times the number of presences in each model) were generated using the random strategy (Thuiller et al. 2013). Model evaluation was carried out by splitting the data; 70% for calibration and 30% for validation, and evaluated using the area under the curve (AUC) of the receiver operating characteristic (ROC) (Swets 1988), and the True Skill Statistic (TSS) (Allouche et al. 2006). Three evaluation runs were made and the mean result extracted. A final model was built without splitting the data, i.e. 100% of the data available was modelled. Thus for each 2 km grid square, projection outputs included a binary score of suitability/unsuitability (i.e. 1 or 0) and mean suitability (a value ranging between 0 and 1000), for each climate change scenario and time period considered.

5.3.3 Integrating dispersal constraints into SDMs

Using the outputs of the SDMs, predictions of future migration of *R. ponticum* under climate change were generated in the R package MigClim (Engler et al. 2012), integrating dispersal and life history parameters specific to this species. Grid-based projections of dispersal were implemented at a cell resolution of 30 m. SDM climatic suitability scores were resampled through bilinear interpolation to match this grid resolution, and then converted into suitability/unsuitability values by specifying a classification threshold using 'binary mode' in MigClim. Data on dispersal and life history traits for parameterization of the MigClim model were obtained from available literature sources. For *R. ponticum* this included a negative exponential kernel with propagule dispersal up to 120 m (i.e. 4 grid cells) distance (Griffin 1994, Harris et al. 2009, Travis et al. 2011); a minimum age of reproductive maturity at 11 years (Cross 1975b, Harris et al. 2009); followed by a logistic rate of increase in propagule production, up until a levelling-off in fecundity around year 40 (Griffin 1994). Occasional stochastic long-distance dispersal (LDD) events are suspected to occur for this species at distances which may exceed 1 km in turbulent air conditions (Shaw 1984, Griffin 1994), such as in mountainous regions – and so LDD was here set in the range of 150-1500 m, at a frequency of 2% once full propagule production potential had been obtained. Output was generated for the most optimistic combination of climate change scenario and ensemble model (B1 – committee averaging), as well for the most pessimistic scenario of climate change and ensemble model (A2 – mean averaging). For both scenarios, results are presented for each climate change time period for each native disjunct region (Spain, Portugal North, Portugal South).

5.3.4 A framework to identify temporally stable microrefugia robust to uncertainties

Climate change microrefugia (which are potentially suited for translocation of *R. ponticum*) were identified based on a high level of accordance in climatic suitability across combinations of different climate change scenarios (B1, A1B, A2), time slices (2021-2050, 2051-2080) and ensemble models (mean, weighed mean, median, committee averaging) (Appendix 7). These combinations therefore afforded a total of 24 different layers (i.e. four ensemble model types within two future time slices within three climate change scenarios) each possessing a binary climatic suitability score (0 = unsuitable, 1 = suitable) predicted per 2 km cell. Based on these layers, we developed a framework (Figure 1.), which was used for identification of robust, and temporally stable microrefugia for *R. ponticum* accounting for statistical and climate change uncertainties. Each cell was summed across these 24 layers, and a raster layer generated containing the result. Cells which possessed high accordance between layers thus exhibited high scores (to a maximum of 24), and we here defined a cut-off level of 83.3 % accordance (i.e. a score of ≥ 20 out of 24) as a reasonable threshold of robustness to uncertainties. High scoring cells (≥ 20 score) were further subject to an aggregation criterion, restricting selection to those which were spatially clustered into groups of 3 or more adjoining cells (i.e. ≥ 36 km^2 total contiguous area – deemed as a minimum desired range size for translocation, based on species-specific ecological considerations). This final output of aggregated cells was thus considered to represent temporally stable microrefugia which are most robust to both climate change and statistical uncertainties, and were converted into polygon shapefiles. Overlap between identified microrefugia and the present occurrence of a target species would therefore signify that robust and future stable sites are already occupied (Fig. 1b), forming the most ideal scenario for conservation. If the species is absent from identified microrefugia, but these possess 'Current' climatic suitability (here predicted from four different ensemble model types as previously specified), such locations represent strong candidates for translocation which could be enacted presently (Fig. 1c). However, if the species is both absent and climatic suitability does not yet exist, these sites represent future microrefugia which will be robust and stable upon their eventual emergence (Fig. 1d). It is hence possible that none to all three microrefugia types (Fig. 1b-d) can be identified and exist simultaneously for species, based on the outcome(s) of this framework.

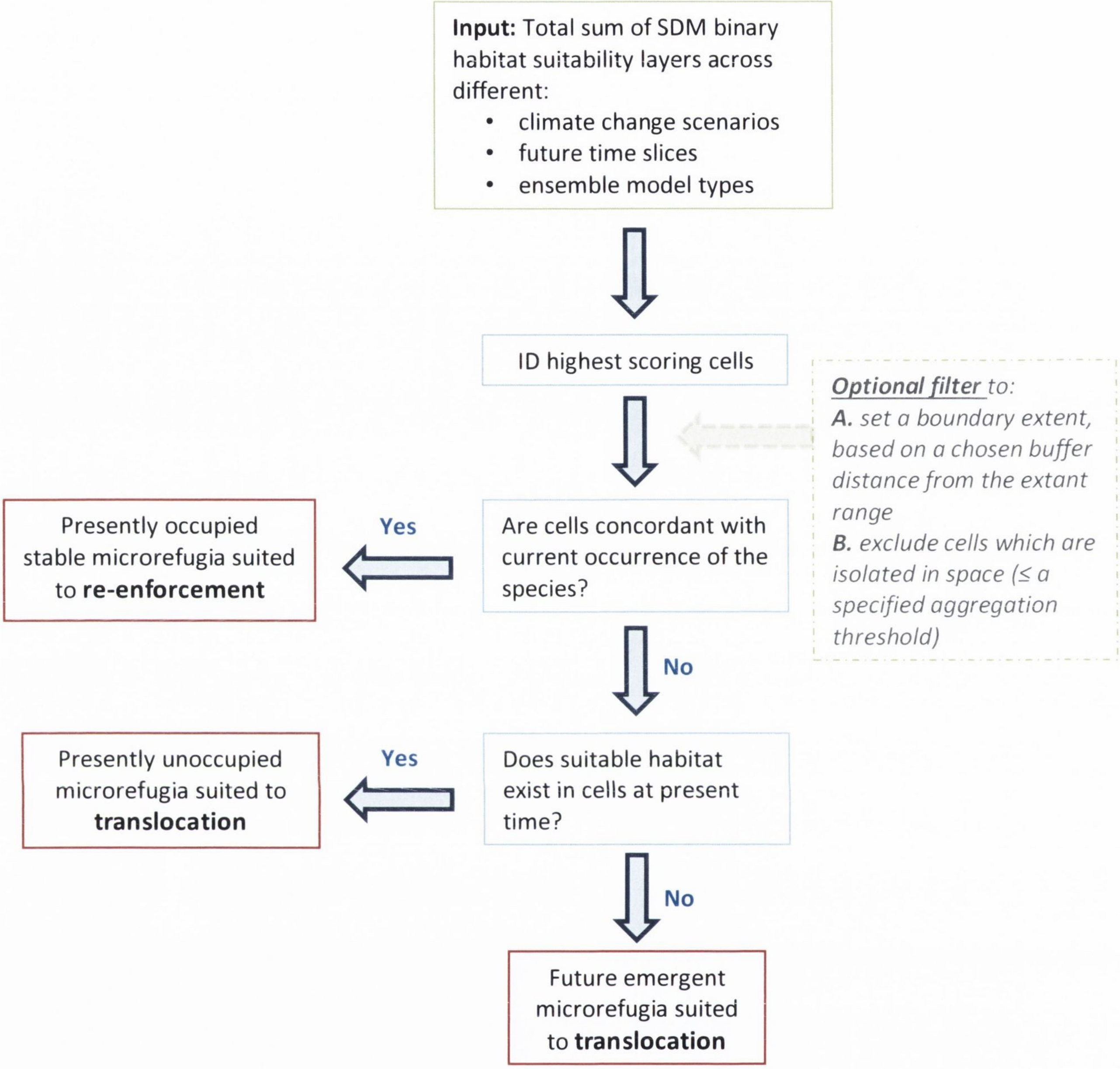

Figure 1. A decision framework for the identification of robust, climate-proof microrefugia potentially suited to translocation, and associated management options. Identified sites thus exhibit high accordance in climatic suitability between the various climate change scenarios and consensus models employed. This framework may thus help guide decision-making taking into account the risk of uncertainties, and given its temporal and spatially explicit nature, can help tackle the questions of 'if, where and when' translocation should/could take place.

5.4 Results

5.4.1 Dispersal-limited migration under climate change

Under the most optimistic scenario of climate change and ensemble model combination (B1 – committee averaging) employed, suitable climate for *R. ponticum* was predicted to have significantly contracted by 2080 in one of its disjunct regions (Portugal South), and have disappeared completely in another (Spain) (Table 1). Regardless of its extent, however, the vast majority of future suitable climate was predicted as non-accessible for dispersal and colonisation by the species (Table 1). While a limited amount of dispersal into areas of future suitable climate was predicted as possible in Portugal North & South; in Spain, it was forecast that by 2050 the species would no longer occupy any area possessing suitable climate.

Under the most pessimistic scenario of climate change and ensemble model combination (A2 – mean averaging), the existence of suitable climate was predicted to have disappeared completely by 2080 from two of the species' disjunct regions (Portugal South & Spain), and have severely contracted in a third (Portugal North) (Table 1). Considering dispersal ability, by 2050, it was predicted that dispersal limitations will have prevented the species from occupying any area of suitable climate across all regions.

5.4.2 Large-scale shifts in future climatic suitability

Within a biogeographical context, it was predicted that the number of areas with suitable climate for *R. ponticum* within its extant range will rapidly decline, as well as contract as a whole within the present day Mediterranean biogeographic zone, while expanding within the Atlantic biogeographic region (Table 2). In some cases by 2050 (but in all cases by 2080) the majority of future climate suitable for *R. ponticum* was thus predicted to exist: A.) outside the current extant and documented historical range of this species; and B.) within geographical areas that fall outside the species' presently occupied biogeographic region. While ensemble models calibrated for the native range performed extremely well here (as judged by model validation); these failed to predict the existence of suitable climate throughout the present entire invasive range of this species throughout NW Europe (0 cells predicted as suitable across all model combinations).

Table 1. Simulated dispersal-limited climate suitability for *R. ponticum* ssp. *baeticum* under progressive climate change. Results indicate the progressive number of cells (at a spatial resolution of 30 m) which possess climatic suitability within each of three disjunct native regions of the species. This simulation was conducted for both the most optimistic and pessimistic climate change scenario/ensemble model combination. Cells were enumerated as: 'total suitable' (total no. cells which possessed climatic suitability), 'occupied under no dispersal' (progressive no. cells in which the species is predicted to occur if no successful dispersal was possible), and 'occupied under partial dispersal' (progressive no. cells in which the species is predicted to occur if some, ecologically feasible, dispersal was possible)

Range	**Scenario period**	**No. cells (B1 scenario/EM ca)**			**No. cells (A2 scenario/EM mean)**		
		Total suitable	Occupied – no dispersal	Occupied – partial dispersal	Total suitable	Occupied – no dispersal	Occupied – partial dispersal
Portugal North	Current	10486649	14	786	2373414	14	806
	2021-2050	14629080	14	2585	2485544	0	0
	2051-2080	11983347	0	1970	718275	0	0
Portugal South	Current	2379798	51	2830	1727140	51	2792
	2021-2050	108753	43	6084	0	0	0
	2051-2080	18008	41	12625	0	0	0
Spain	Current	5084667	101	6088	4154303	101	6046
	2021-2050	5160	0	0	0	0	0
	2051-2080	0	0	0	0	0	0

Table 2. No. 2km cells possessing suitable climate for *R. ponticum* ssp. *baeticum* within its extant area of distribution, and per biogeographical zone on the Iberian mainland. Mean climatic suitability scores (ranging from 0-1000 ± SD) are presented in parentheses.

Time/Range	**Extant range**†	**Mediterranean zone***	**Atlantic zone***
	No. 2km^2 cells		
Current			
Current (EM ca)	759 / 780 (± 264)	6894 / 45 (± 154)	742 / 69 (± 153)
Current (EM mean)	486 / 603 (± 241)	1591 / 42 (± 106)	25 / 67 (± 97)
B1 scenario/EM ca			
2021-2050	293 / 405 (± 303)	3756 / 32 (± 123)	2368 / 158 (± 226)
2051-2080	109 / 187 (± 235)	2086 / 15 (± 83)	3212 / 208 (± 246)
A2 scenario/EM mean			
2021-2050	0 / 163 (± 174)	19 / 24 (± 63)	121 / 145 (± 159)
2051-2080	0 / 44 (± 36)	1 / 18 (± 37)	69 / 140 (± 138)

†The present extant range was delimited by georeferencing distributional data from Perez Latorre & Cabezudo (2006) and Magos Brehm et al. (2008)

*Confined to mainland Portugal and Spain only

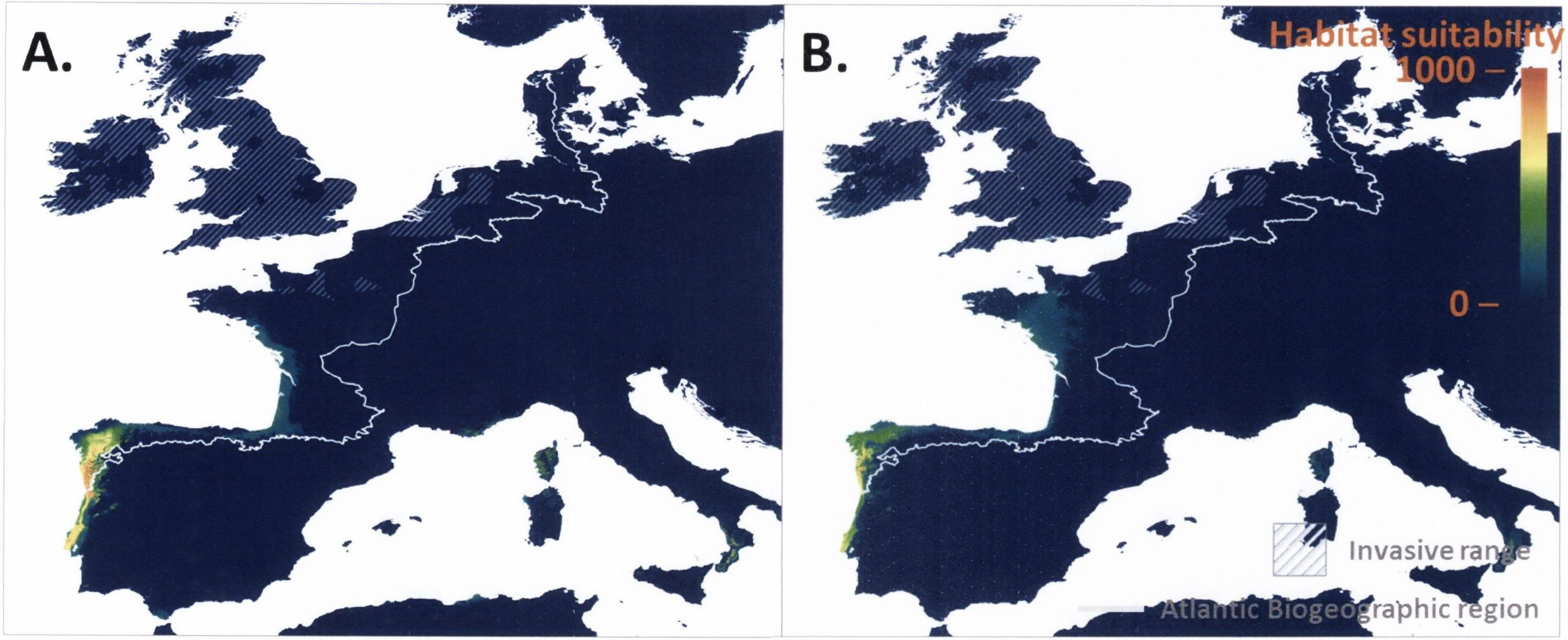

Figure 2. Areas of climatic suitability for *R. ponticum* ssp. *baeticum* predicted for the 2051-2080 time slice, under A.) the most optimistic climate change scenario and ensemble model combination (B1 scenario – ca ensemble), and B.) the most pessimistic climate change scenario and ensemble model combination (A2 – mean ensemble). In both cases the majority of suitable climate is predicted to shift into areas of the present Atlantic biogeographic region.

5.4.3 Identification of robust climate change microrefugia

The framework developed and applied as part of the current study was successful in identifying candidate sites potentially suited for translocation of *R. ponticum* for conservation purposes. A small number of 2 km cells in N Portugal and NW Spain (i.e. outside of the species' historical distribution/present biogeographic zone) were identified as microrefugia most suited for potential translocation. These cells indicated a relatively high level of accordance (83.3 %) in prediction of climatic suitability across the 24 different layers considered (accounting for different combinations of climate change scenarios, time slices and ensemble model types). These areas were thus deemed to be temporally stable under a range of climate change scenarios and robust to the statistical uncertainties associated with the ensemble of modelling techniques. However, as the species does not presently occupy these cells, and these do not possess climatic suitability under 'current' conditions, these sites therefore represent future emergent microrefugia (i.e. Fig 1d).

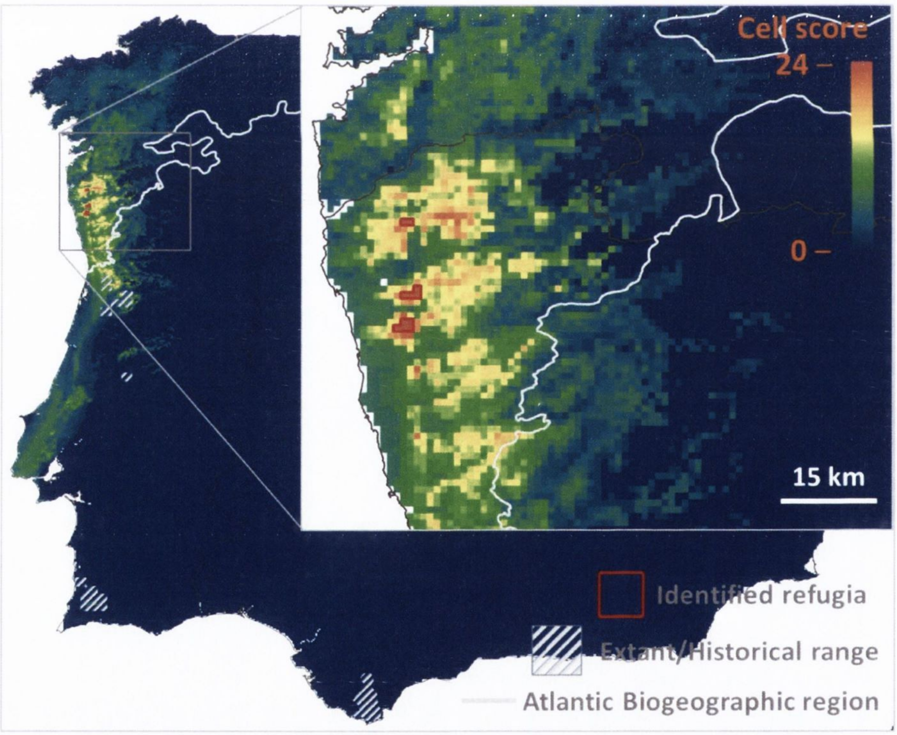

Figure 3. Identified climate change microrefugia for *R. ponticum* ssp. *baeticum* potentially suited for translocation. Higher cell scores indicated areas with high accordance in climatic suitability (between the various climate change scenarios and consensus models employed). Efforts aimed at conserving this taxon from extinction by means of assisted colonization would hence most likely require translocation into microrefugia outside of the species' extant (and known historical) range, and present biogeographical region of occurrence

5.5 Discussion

5.5.1 Migration into future suitable climate under climate change

Our results suggest imminent climate-induced extinction of *R. ponticum* ssp. *baeticum* in its native range due to loss of suitable climate in areas of current occupation, coupled with an inability to migrate into future suited areas. Only under the most optimistic scenario was it predicted the species could occupy small areas of future suitable climate within the vicinity of its extant Portuguese range – whereas no occupation of future suitable climate was predicated anywhere in the most pessimistic scenario. However, as the mean ensemble model (EM) technique (which represented the most pessimistic predictor of climatic suitability) showed the best specificity for predictions, there is reason to believe that this EM may be more realistic as a scenario. However, there was nonetheless consensus between scenarios in prediction of range collapse in the species' disjunct Spanish range, where it is Red-listed nationally (Blanca et al. 1999). While our simulations of propagule dispersal in this species assumed full rates of germination and transition between life stages, in reality it is known that successful recruitment of new juveniles is extremely rare in the native range (Mejías et al. 2002). An almost categorical lack of natural recruitment would therefore best equate to our highly conservative predictions of migration ability based on 'no dispersal' (Table 1), and further affirm the likelihood that climate change impacts will follow a very worrying path, closer to the most pessimistic of trajectories.

Explanation as to why SDMs performed very well according to model evaluation, yet did not predict suitable climate in the invaded range, may relate to the high specificity of *R.p.* ssp. *baeticum* to relict habitat in the native range, upon which models were calibrated. The species is thus seen to possess 'hidden plasticity', which is not apparent from the limited range of environmental conditions occupied in the native range. While on one hand such hidden plasticity may lead to failure in predicating invasive capacity in novel geographic areas, this would also lead to more conservative estimates of climatic suitability and areas suitable for translocation.

5.5.2 The invasive alien/threatened native juxtaposition

Given the very uncertain survival of *R. ponticum* based on evidence presented in this study, this species would arguably qualify as a candidate for translocation in accordance with current guidelines (IUCN/SSC 2013), or at least consideration towards such on a precautionary basis. However, as is the case for other threatened IAS identified (Appendix 6), there exists a paradox in how best to approach practical conservation of such species, and what role (if any) invasive populations may play, both now and under future climate change. While eradication may for now continue as a goal within the introduced range, if risk of native extirpation continues to intensify (as predicted here for *R.p.* ssp. *baeticum*), at some point the appropriateness of this strategy must be considered within a broader perspective. For instance, this may include the reintroduction of individuals or propagules back into the native range, and/or acceptance of these taxa as 'climate-endangered' species (Thomas 2011). The latter may not come with much pre-conceived choice in the circumstance that future suitable habitat may just simply no longer exist in the native range, or reintroduction otherwise proves unviable.

Heated debate currently surrounds the ethical desirability of translocation of potential climate-endangered species, as well as the usefulness of a 'native' versus 'alien' duality in a future world where extinction due to migrational lag may be much more common-place (Warren 2007, Ricciardi and Simberloff 2009b, Warren 2009, Minteer and Collins 2010, Schwartz et al. 2012). Hence given their unique juxtaposition, threatened IAS can offer more than just hypothetical examples in this context. Could a paradigm shift towards acceptance of such taxa as past-translocated (as opposed to purely exotic) species represent a realistic proposition, either now or in the future? How climate change will impact threatened IAS in their invasive range may ultimately steer such considerations, as future habitat suitability may be altered to levels which safely contain invasive spread, or even lead to range contractions here.

5.5.3 Narrow endemics and challenges to translocation

While the theoretical aim of translocation is straight-forward, conceptual challenges are posed in complex cases such as for narrow endemics like *R.p.* ssp. *baeticum* – which even in present times may occupy regionally rare microclimates already under siege. Such a scenario represents a very significant challenge for this and perhaps many other similarly threatened relict endemics – in that the present extant range (however small) may also coincide with the known historic range for these species e.g. due to long-term isolation in relict habitat, or their relatively recent evolutionary emergence (Lavergne et al. 2004, Hopper 2009). Therefore translocations which are implemented outside of these areas are deemed as 'introductions', and this practise is currently not accepted by some authors as a valid strategy (Ricciardi and Simberloff 2009b, Ricciardi and Simberloff 2009a). However, as evidenced from our results and other past findings on this species (Erfmeier and Bruelheide 2010, 2011), translocation of *R.p.* ssp. *baeticum* into areas both outside its present biogeographic zone and extant/historic area of occurrence represents the most feasible chance for its survival.

While guidelines suggesting translocation should be restricted to within a species' present biogeographic zone are well founded (on the basis of preventing unintended impacts on recipient ecosystems); our findings indicate that rigid interpretation of this principle will undoubtedly hamper efforts for conservation in some cases. It is arguable that such a criterion possesses little relevance for narrow endemics like *R.p.* ssp. *baeticum* that occupy relict microclimates; which in their very nature contrast sharply with regional climate typical of the prevailing biogeographical zone. Furthermore, this would not make sense when analogous areas possessing long-term stability may be readily identified in adjacent biogeographic regions. Indeed such areas may very well exist within short distance of a species' extant range, as was observed here for *R.p.* ssp. *baeticum*.

Based on the above, we make the strategic recommendation that where necessary; consideration must be afforded to implementing translocation outside of a species' present biogeographical zone. We call for this in a bid to foster less reliance on present biogeographic delineations generally, as a critical guiding principle for translocation; as such delineations do not prove useful when dealing with projections of future climate change (Williams et al. 2007, Mahlstein et al. 2013). Some authors have suggested broadening the definition of a species' 'historic' or 'indigenous' range to include any area once occupied by a species; regardless of how far in the past this may be (Jørgensen 2011). While it is apparent that care must underpin any such interpretation of historic (or 'historical') range (Dalrymple and Moehrenschlager 2013); successful identification of these areas could at least guide and enhance the legitimacy of translocation outside of a species' present biogeographical zone – provided a genuine need has been established and that historical areas do possess adequate and future-stable climate.

5.5.4 Identification of robust climate change microrefugia

We here developed a framework for the identification of stable climate change microrefugia that is both robust to statistical uncertainty (based on consensus between ensemble model types), and accounts explicitly for uncertainty between climate change scenarios. The framework is highly flexible to adaptation for additional inputs, which may depend on the level of robustness sought. For example, we here employed 24 different climate change scenario layers derived from one global circulation model (GCM) and two future time periods, but this could be expanded to consider accordance between multiple other GCMs and finer temporal resolution, as desired.

While this framework is relatively straightforward in principle, we believe it represents a powerful tool to assist practical identification of climate change microrefugia and decision-making for purpose of conservation translocation. As translocations are generally considered the last line of action for averting species extinction, there is therefore significant pressure on practitioners to make informed and timely management decisions. However, given the relative urgency and extreme uncertainty at which such decision-making is frequently undertaken, proper consideration has not always been afforded to the long-term suitability of recipient sites, which may prove all the more tactless where species decline is (at least in part) climate-induced. Our framework can therefore assist for such purposes, and given its temporal and spatially explicit nature, can help tackle the questions of 'if, where and when' translocation should/could take place. While candidate sites may not be numerous (in comparison to use of a single modelling technique or climate change scenario), the crucial benefit is that these possess high temporal stability and robustness to uncertainties, and may hence minimize risk of failure in the short and long-term.

Demonstration of this framework applied to *R.p.* ssp. *baeticum* revealed that this species does not presently occupy (or in all likelihood can migrate into) long-term stable microrefugia. We were successful in identifying several small areas of putative areas of future stable microclimate potentially suited for translocation by the end of current climate period (i.e. by ca. 2020). While the temporal resolution of climate periods used in this study was rather coarse, using shorter consecutive time intervals (e.g. 5-10 year periods) could afford greater accuracy as to forecasting when stable microrefugia may emerge and translocation ideally be initiated. Notwithstanding a more precise timeline, knowledge of where stable habitat is predicted to emerge for this and other climate-endangered species is of relevance for contingency planning in the present-time (Hunter 2007), before climatic suitability in the extant range is predicted to undergo more serious decline. If under further scrutiny these identified putative microrefugia are selected as recipient sites for translocation, management action may already be implemented in advance of planned establishment, and could include development of microhabitat and other abiotic or biotic niche components to support this.

Chapter 6

General Discussion

"Some chemists, having synthesised a few compounds
believe themselves to be better chemists than nature,
which, in addition to synthesising compounds too numerous
to mention, synthesised those chemists as well."

– Marcel Jaspers

6 General Discussion

The general aims of this thesis were two-fold; firstly, to further knowledge on the topic of toxic nectar in plants, through investigations on a model species (and associated taxa) of its spatial pattern of occurrence, potential abiotic influences, and evolutionary ecology of adaption to mutualists at both micro and macro scales (Chapters 2-4); and secondly, to assess climatic suitability for *Rhododendron ponticum* under future climate change, together with projections of the species' migrational capacity, as a model scenario to inform best-practice in conservation translocations (Chapter 5). In this chapter, I explore the wider relevance of the main research findings, and place these within larger perspective of the fields of chemical ecology, and conservation biogeography. To conclude, I explore a number of future potential research directions, which may capitalise and build on the outcomes of this research.

6.1 Summary and synthesis

6.1.1 Toxic nectar and multispecies interactions

Despite an assumed importance in regulating interactions with mutualists, there has been little fundamental investigation into how expression of toxic (or deterrent) compounds in nectar may vary in spatial terms (Kessler et al. 2012). In Chapter 2, large spatial heterogeneity in expression of GTX in *R. ponticum* nectar was uncovered. Significant geographic variation was observed between its native and invasive range, but with notable small-scale gradients also apparent between individuals within populations. The emergent picture from these (albeit few) studies therefore suggests that variation (whether through consequence of plasticity due to abiotic influences, or adaptation), may be expected to be a relatively common feature amongst toxic-nectar producing species. However, many more such studies are required, across diverse species and classes of secondary metabolites, before meaningful generalisations can be made.

It is clear that to-date very little consideration has been afforded on the significance of intraspecific variation in toxic nectar, and what implication this may hold towards its many hypothesised adaptive functions in plants. The importance of such variation should also be acknowledged in future studies seeking to examine bioactivity of nectar toxins. Affirming the existence (or lack thereof) of an ecological function/ bioactive effect based upon single-concentration treatments can considerably oversimplify what in fact may be complex patterns of variation (and variation in function) in the wild, as evidenced by our findings. What has also commonly been missing from deliberations on the ecological significance of toxic nectar, beyond the topic of variability, is the question of the absolute level of compound concentration in nectar. As discussed in Chapter 2, it is now clear that secondary metabolites are expressed at very different concentrations in plant nectar, ranging from comparatively low and almost trace levels, to high levels of expression – such as those observed for GTX I (Chapter 2) and other compounds (London-Shafir et al. 2003, Boros et al. 2010, Cook et al. 2013, Gosselin et al. 2013). Such high levels of expression may even come close to, or indeed exceed, those in other plant tissues such as leaves, presumably expressed for defensive purposes. Hence, consideration must be afforded to aspects of both concentration and variability in a conjoint manner, in future assessments of the potential ecological role of toxic nectar in floral interactions.

It is widely held that, for many species, nectar constitutes the single most important trait governing plant interactions with mutualists (Heil 2011). The question as to how nectar can be adapted by invasive non-native species, as part of the colonization process, has only scarcely been touched upon to-date (Stout et al. 2006, Ollerton et al. 2012). Hence, few studies have sought to compare nectar characteristics across the native and non-native range of introduced species, let alone nectar secondary metabolite content. Building upon the patterns observed within Chapter 2, the objective of Chapter 3 therefore was to examine how selective pressures within the invasive range of *R. ponticum* may influence the expression of toxins within nectar. I provide in this study the first empirical evidence of natural selection acting on toxic nectar, and link this to selective pressures applied by pollinators – which, in contrast to the native range, have seemingly acted to reduce nectar toxin levels in invasive plants. I also found that within the invasive range, phenotypic correlation in toxin expression between tissues (leaves, flowers, nectar) have become completely uncoupled; in contrast to the situation in the native range. This functional uncoupling has evidentially enabled invasive plants to surmount the ecological and evolutionary constraint posed by non-tissue specific expression of defensive compounds. This novel adaptation has hence permitted pollinator-mediated selection to act independently on nectar GTX, while bearing no influence on GTX levels in leaves, where these compounds served as an effective chemical defence.

Previous studies have reported how pollinators may, through consequence of selecting on visually attractive floral phenotypes, also indirectly influence compounds which serve a defensive function against herbivores in plants (Simms and Bucher 1996, Irwin et al. 2003). However, the main findings from Chapter 3 bear large importance, as they demonstrate for the first time the direct occurrence of pollinator-mediated selection on secondary defence chemistry in plants. To-date, investigation of selective pressures on plant chemical defence traits have almost exclusively focused on plant-antagonist interactions (Ibanez et al. 2012). It is commonly known, for instance, that plants must strive to strike a balance in their phenotypic expression of chemical defence compounds; as opposing pressures from specialist and generalist herbivores drive selection for either decreased or increased expression, respectively, depending on the relative local abundance of these guilds (Lankau 2007). To the general study of the chemical ecology of plant secondary metabolites, we must evidentially further add to this equation, therefore, the potential for direct selection by mutualists on plant chemical defence.

I progressed from the topic of microevolution in Chapter 3, to a wider macroevolutionary scale of focus on toxic nectar in Chapter 4. The findings from this study (Chapter 4), which focused on a broad sample of species of *Rhododendron* that exhibited a diversity of pollination syndrome types, provided further evidence in support of the ability of plant mutualists influence the expression of toxic secondary metabolites in plants. This finding adds to the few other studies on the topic of macroevolution of toxic nectar (Adler et al. 2012, Manson et al. 2012), which have similarly considered and/or implicated pollinators as possible driving forces shaping the expression of toxic nectar. The novelty of findings from Chapter 4, however, relate to how species which have adapted to pollination through different pollen vectors (such as bee, bird, butterfly or moth pollination), hold different likelihoods of exhibiting toxic nectar – as degree of floral specialisation was negatively associated with the prevalence of toxic nectar amongst species.

From a larger perspective, the emerging picture from these (Chapter 3 and 4) and past studies indicate there are grounds to suggest that toxic nectar may in fact represent an evolutionarily primitive character state in plants. Indeed, it has been observed more often (Adler et al. 2006, Halpern et al. 2010, Cook et al. 2013, Lohaus and Schwerdtfeger 2014) than not (Manson et al. 2012) that plants are constrained, in an evolutionary sense, through phenotypic correlation between leaves and nectar in expression of secondary defensive compounds. Such a scenario, however, does not necessarily preclude the possibility that toxic nectar may perform an adaptive function in plants within contemporary ecological settings. Similar to how triterpenoid resins are thought to have evolved as floral rewards from an original primary defensive function (Armbruster 1984, 1997); toxic nectar may have accordingly subsumed an adaptive role in floral interactions (whether as beneficial to mutualists, or as a defence against antagonists, or both). This scenario may also be construed as analogous to the evolution of specialisation by phytophagous insects – in which specialised herbivores have successfully co-opted the use of (what have originally been) toxic or deterrent defensive chemicals produced by plants (Smallegange et al. 2007, Talsma et al. 2008). On the other hand, it may therefore equally be expected that 'loss' of toxic nectar represents an evolutionary advanced character in plants; resultant, for instance, as an adaptive response from rapid shifts between functional groups of pollinator types (Chapter 4), or as part of greater 'generalization' of pollination syndromes under sustained pressure from pollen limitation (Chapter 3). As a whole, the combined findings from this thesis (Chapters 2, 3 and 4) therefore add to our ability to explain why some species (or higher taxa) may express toxic nectar, while other phylogenetically-related congenerics apparently do not. As noted in Chapter 3, the generality of such findings in nature may in fact be broad, given the wide distribution of toxic nectar amongst angiosperm families.

6.1.2 Translocation of climate-endangered species

In Chapter 5, I collated a list of invasive alien species (IAS) which are also known as threatened (to various extents) in their native range. Focus in particular was afforded to *Rhododendron ponticum*. The main findings emergent from this study, as a whole, suggest the distinct possibility of climate-induced extinction of *R. ponticum* through A.) loss of suitable climate in areas of its current occupation, coupled with B.) an inability to migrate into areas of future suitable climate. Under standard criteria, the species would arguably qualify as a target for translocation in order to avert (what for all intensive purposes) appears to be impending extinction in the wild in the not-so-distance future.

Several options were explored in Chapter 5 on how to best deal with *R. ponticum*, and other potentially climate-threatened IAS, in relation to future conservation needs. In particular, deliberation was afforded on what role (if any) invasive populations may play in future efforts to conserve these species in their native range, and if at some future point (if current trends continue) the appropriateness of the goal of eradication in the non-native range will need to be brought into question. An alternative approach for these IAS may be acceptance of these as a 'climate-endangered' species in the non-native range, akin to climate refugee species *sensu* Thomas (2011). Such a topic undoubtedly raises concerns from the point of view of environmental ethics, and may indeed bring into question the conventional paradigm of mainstream conservation, which is based firmly on the concept of *in-situ* preservation of species (Fiedler and Jain 1992). However, as discussed in Chapter 5, acceptance of IAS as climate refugee species may in reality be imposed rather than elected.

Based on a case-study of *R. ponticum* within its native Iberian range, I proposed a framework which may allow for the general identification, in spatial terms, of robust and species-specific climate change refugia potentially suited for translocation. It was revealed that guidelines which define the indigenous range of a species in a narrow sense would not make much sense when applied for narrow endemics. In the case of *R. ponticum*, it was revealed that even translocation into identified candidate sites a relatively short distance away from its present area of occupancy would technically equate to translocation outside of its indigenous range. Some suggestions were therefore made in order to define best-practice in such circumstances, which include; firstly, ideally minimising the distance between areas of a species' current occupancy and where translocation should be enacted in suitably identified sites; and secondly, a departure from a strict reliance on discrete biogeographic regions, as a guiding principle to legitimatise translocations of narrow endemics which are climate-endangered.

A notable discrepancy between *R. ponticum* and other IAS, however, is that pollen records indicate a pre-historic distribution of this species throughout much of the European continent, including Ireland (Cain 1944, Jessen 1948, Jessen et al. 1959, Martinetto 2009), where it is now considered a non-native. Whether or not this former distribution could, or should, force reconsideration of the 'invasive' label presently assigned to populations of *R. ponticum* from NW Europe is hence a subjective matter, and centres around how a species' 'indigenous' range ought to be defined (Jørgensen 2011, Dalrymple and Moehrenschlager 2013). Two logical arguments in particular, put forward by various authors on the topics of translocation and invasive species, may perhaps better frame the position of *R. ponticum* – these being;

- firstly, that the validity of a native/alien construct as an organising principle in conservation can be expected to fade somewhat under climate change (at least in its strict sense), as we regardless should anticipate large ecological change. If major biodiversity-climate disequilibria are to be prevented, this change should rightly feature the addition of many novel species (via their natural migration) to the flora and fauna of regions, accompanied by the emergence of novel species assemblages in place of those currently found. In other words, ecological communities as we know them should not be expected to stay the same, and it must be accepted that even 'legitimate' neophytes will inevitably displace and out-compete older-established species less adapted to a future climate;
- and secondly, that a 'damage criterion' (Warren 2007, 2009) could perhaps better serve as an organising principle in place of a native/alien polarity. Hence, whether a given species is native or alien to a given region would not exactly hold any great significance *per se*; with emphasis instead placed on how likely any species is to negatively impact on overall biodiversity and ecosystem function. In fact, in some British woodlands it is indeed native species, and not aliens, which are the "thugs" (Marrs et al. 2010). From this perspective, the goal of management, in striving to maximise biodiversity (or more realistically, the ecosystem services which flow from it), should be to control any invasive species regardless of native or non-native status (such as in the example of bracken (Marrs and Lowday 1992)), and not the eradication of non-native species purely on ideological grounds alone.

Reconciling these views with the reality of the situation for *R. ponticum* may be challenging, however. On one-hand, the ecological damage caused by this species in Ireland, in particular, may be large – such as in disruption of natural successional cycles in Oak woodland (Cross 1981, Kelly 1981, 2005). However, this capacity to interrupt forest regeneration is also known from the species' native range in Turkey (i.e. for *R.p.* subsp. *ponticum*), and is hence not a unique feature of non-native populations (Yildiz et al. 2009). On the other hand, there is now evidence to support that *R. ponticum* in Ireland is differentiated from its Spanish ancestors beyond what may be explained by phenotypic plasticity or introgression with congenerics (Erfmeier et al. 2011), and exhibits unique post-invasion adaptations to its local environment (Chapter 3). Within the non-native range this species may in fact provide valuable habitat and food resources for other species (Malo et al. 2012), and in particular receives a high visitation rate from pollinators (Stout et al. 2006, Stout 2007a, Dietzsch et al. 2011).

While *R. ponticum* is subject to heavy control measures at present, in reality this is not likely to achieve anything close to complete eradication, due to the various difficulties facing such interventions (Wong et al. 2002, Dehnen-Schmutz et al. 2004). Given the notable climatic suitability of NW Europe for growth of this species, a case could be argued that under different circumstances *R. ponticum* may have naturally migrated to such latitudes – as there is always an element of lottery involved in which species successfully migrate and persist over time, and which do not. Interestingly, other Ericaceous species, such as *Erica mackaiana and E. erigena*, are also known to have been present in Ireland during inter-glacial times; with *E. mackaiana* in fact identified from the same paleo-deposits alongside *Rhododendron ponticum* (Jessen 1948, Jessen et al. 1959). Yet the distribution of both these *Erica* species in contemporary times is disjunct between Ireland and the Mediterranean (Nelson 1982, Webb 1983), which may support that it is only down to chance circumstances that *R. ponticum* has not been able to distribute likewise. The *in-situ* survival of these *Erica* through the last glaciations has been considered (Webb 1983), however evidence suggests (at least for other similarly disjunct species) that postglacial recolonization represents a more likely scenario (Beatty and Provan 2013). The contrasting fates of these Ericaceous species in modern times is therefore ironic, as despite their sympatric pre-historic distribution in Ireland, one is now perceived as an aggressive non-native invader, whilst the others are cherished as rare species.

6.2 Methodological considerations

Studies in this thesis drew upon a wide variety of methods from across a range of disciplinary fields, and featured use of phytochemical, evolutionary, biogeographical and ecological techniques. I considered this wide focus integral to addressing many of the questions here posed. Many of the methods employed and experimental designs followed well-established protocols, and the success of their utilization could be clearly and accurately evaluated. Others, however, followed more cutting-edge techniques whose evaluation is still subject to some degree of uncertainly, or limitations were encountered in experimental design (such sample sizes) and had to be contended with. As an exercise that may be useful to guide future such investigations, I here evaluate some of these methods employed, in terms of both the challenges and successes that were met.

It was sought in Chapter 2 to examine geographical variation in toxin levels expressed in nectar. Additionally, a number of abiotic variables and factors were measured to see how such conditions could possibly account for the spatial variation subsequently observed for this trait. The limitations of such an assessment based on wild populations was noted, however, as observed co-variation of nectar toxins was made only on the basis of association, and not causation, which could only have been afforded through experimental manipulations. Thus, while several abiotic variables in particular appeared to be associated with variation in nectar toxins as a whole across the native and invasive range of *R. ponticum*, it must nonetheless be considered that genotype-environment interactions could also play a significant part in observed patterns, and could only be untangled through cultivation or transplantation experiments similar to as Erfmeier and Bruelheide (2010). This scenario did not detract from overall findings however, as other lines of evidence (in Chapter 2, but ultimately in Chapter 3) helped me establish that changes in phenotypic expression of nectar toxins in the invasive range were most likely due to adaptive causes beyond (though not necessarily excluding) the occurrence of environmentally-induced variation.

Chapter 3 concerned the measurement of natural selection, and its potential biotic causes, on GTX levels in leaves, flowers and nectar of *R. ponticum*. A particular concern, which was also discussed within the chapter, was of the adequacy of sample sizes for detecting effects, if present. Currently there remains a question of the best way to quantitatively compare the 'strength' of natural selection (such as between species, within species across geographical ranges etc.) in a way that is objective (Matsumura et al. 2012). While the findings from this chapter reveal a moderate strength of selection operative on tissue toxin content, it is likely however that this would not have been detected if sample sizes were much smaller, due to inadequate statistical power. Otherwise in this study, care was taken to ensure a balanced argument in linking biotic pressures as the cause of observed selection on GTX levels. If plants which exhibit high GTX levels also experience pollen limitation, then pollinators are implicated in selection. However, what is not clear from established knowledge to date on nectar GTXs (Tiedeken et al. 2014) is if pollinators indeed possess the ability to consciously select or avoid low or high toxin-containing plants, or perhaps are just avoiding these for some other reason (although data collected, but not presented, indicated that nectar quality at least did not vary in some other ways, such as water/dried matter content). Regardless of whether selection by pollinators was imposed consciously or not, the end result was the same, in that selection was exerted against nectar toxins.

One of the main challenges in assessing the taxonomic distribution of nectar GTXs in the genus *Rhododendron* (Chapter 4) was in the potential occurrence of false negatives, i.e. that GTXs were recorded as absent in instances in which they were present. While I devised a method aimed at explicitly avoiding (such as sampling as many individual plants from species as possible), the possibility of the occurrence of false negatives nonetheless remained. However, as noted in Chapter 4, these were at least likely to have been taxonomically random, and therefore not of large consequence of overall patterns. In theory, further steps could potentially have been taken to affirm the non-toxicity of Vireya nectar in particular, which appeared devoid of nectar GTXs. However, the question of how to do this in practise would arise, in that a test organism for bioassays would have been difficult to obtain, or not suited for such a test. For instance, the use of bees as a test subject to examine toxic nectar has been commonly used elsewhere (Elliott et al. 2008, Manson et al. 2010, Koehler et al. 2012, Cook et al. 2013, Wright et al. 2013), however, Vireya nectars are in theory adapted to the nutritional needs of birds (e.g. in their more dilute, watery constitution), as well as other specialised pollinators. Hence, in using this nectar with bees, it would be difficult to separate out the cause of any observed negative effects, as these could equally be due to nutritional inadequacy of this nectar as a food source, as well as the presence of undetected GTXs, if indeed present.

There is a drive in current times to build on from the use of static species distribution models (SDMs) towards a greater integration with more dynamic predictions of dispersal (Engler et al. 2012, Franklin et al. 2014). While such an approach was undertaken in Chapter 5, a decision was undertaken to purposely exclude landscape barriers into these spread models (i.e. any land-cover type not potentially colonisable, such as agricultural, urban, grassland etc.). The reasons for doing so were to test a scenario in which all landscape types, if possessing suitable climate, could be colonized once dispersed into. Accordingly, this assessment afforded a highly optimistic representation of how *R. ponticum* may disperse under future climate change. The utility of this approach, in examining optimistic (and/or contrasting pessimistic) scenario types, equates to a useful way of simulating processes which naturally possess a high degree of uncertainty. Due to its straightforward concept and associated language, this type of approach may also translate well in aid of decision-making processes, and has been successfully used as such (Lempert et al. 2004). However, it must also be acknowledged that such an optimistic scenario may be far removed from the realistic challenges which face the species, in migration into future suitable climates. However, even under these 'ideal' conditions, in which intervening landscapes do not form a barrier for dispersal, it was observed that very little such dispersal occurred regardless. I therefore considered this finding (based on an optimistic scenario) to be of greater value than assessment of more 'realistic' scenarios which integrated landscape barriers, as most likely, these would only have served to demonstrate the impossibility of any dispersion at all.

6.3 Future research directions

6.3.1 Toxic nectar and multispecies interactions

In addition to contributions from the field of pollination ecology, future investigations on toxic nectar will continue to benefit from drawing on parallels with plant-herbivore interactions, in terms of both hypothesis testing and methodological techniques. As emergent from the studies described in this thesis (and in particular Chapter 3), it is now clear that a holistic, whole-plant view is required which better integrates plant pollination systems within a multispecies approach. Such integrated approaches will be required in attempts to further our understanding of the ecological significance of toxic nectar and selective pressures exerted from plant mutualists and antagonists, embedded as a whole within the context of plant chemical defence. Some future research directions which could capitalise and build-on the research undertaken here include:

- **Invasive species**: Selection pressures experienced by invasive species in the exotic range can differ markedly from those in the native range; a point which has justified the wide use of invasive species as models in chemical and evolutionary ecology (Richardson et al. 2000, Harvey et al. 2010, Macel et al. 2014). The comparative study of toxic nectar in a broader sample of invasive species may therefore build on findings here (Chapter 2), and reveal whether there is generality in such patterns. Various hypotheses may be put to the test, which (albeit partially overlapping in their predictions) could include: evolution of increased competitive ability (EICA), novel-weapons, and shifting defence hypotheses (Callaway and Ridenour 2004, Joshi and Vrieling 2005, Cappuccino and Arnason 2006, Doorduin and Vrieling 2011). It would be particularly interesting in this context to test whether increases/decreases in leaf chemical defence concur with patterns from nectar.
- **Microevolutionary ecology**: Given the common occurrence in many species of phenotypic correlation in expression of secondary metabolites across tissues, understanding which biotic drivers exert selection on these, and under what circumstances, will continue to represent a topic of large relevance in understanding the evolutionary ecology of chemical defence, including toxic nectar. Assessment of natural selection within a path analysis framework (as employed in Chapter 3) can provide an invaluable tool in such studies, though to-date remains very much underutilised (Scheiner et al. 2000, van Tienderen 2000). Critically, such an approach possesses the flexibility to account for direct and indirect interactions, such as may occur between plants, antagonists and mutualists.

- **Macroevolutionary ecology**: At the macro scale, charting the evolution of genes associated with toxic nectar in 'deep time' may allow greater insight into its origin within and between certain taxonomic lineages; and therefore definitive answers as to whether this trait represents a primitive state, or is an evolutionarily advanced character in plants.
- **Physiology**: The suite of secondary metabolites expressed in nectar may only constitute a sub-set of those produced by a plant, which has lead to the belief that this selective 'filtering' reflects an adaptive function of toxic nectar (Manson et al. 2012). However, it remains apparent how little is known on the origins and means by which secondary metabolites are expressed in nectar. Further exploration at the genetic level of the apparently novel physiological adaptation exhibited by invasive plants of *R. ponticum*, which has served to reduce or block the phenotypic expression of GTX I in nectar, would undoubtedly prove profitable.
- **Hereditability**: Despite its many assumed adaptive functions, and that selective pressures may be operative on toxin levels in nectar; it nonetheless remains to be demonstrated that such a trait is indeed heritable. A quantitative genetics approach could therefore shed light on the extent to which plants can respond to selection on toxic nectar, and the rate at which this trait can change over time. The heritability of many nectar characteristics remains a little investigated topic generally (Mitchell 2004).
- **Botanical occurrence**: The botanical occurrence of toxic nectar amongst diverse plant families is notable in its extent. Studies as conducted in Chapter 4 are rare, however, and it may nonetheless be possible that we are still only 'scratching the surface' in terms of how prevalent this phenomenon is in angiosperms generally. A much broader taxonomic sampling is thus required, moving beyond infamous toxic species; as to-date these appear to have received disproportionate attention. It is note-worthy that there remain few examples of species thought to definitively lack deterrent or toxic secondary metabolites in nectar. Whether this signifies that we have just been looking in all the right places, or not, remains to be seen.

6.3.2 Translocation of climate-endangered species

The case-study presented in this thesis on *Rhododendron ponticum* allowed insight into the future conservation challenges which may face this species, as representative of other invasive alien species (IAS) which are to some extent threatened in their native range. Future research directions are potentially many. For instance, performing detailed analyses for other such IAS could shed more light on these species in particular, similar as was performed for *R. ponticum*. Wider application of the framework developed here, for identification of robust climate change refugia, could also be of large value. For *R. ponticum*, several small areas potentially suited for future translocation were tentatively identified. Thus, assessment of how this framework performs for additional species would be required, in advance of promoting its wider use as a general tool for conservation.

6.4 Concluding remarks

Our knowledge on the remarkable diversity of secondary metabolites in nature is ever-expanding, alongside the complex ways in which these are utilised by plants, as key traits governing interactions with both co-evolved and generalist mutualists and antagonists alike. However, how anthropogenic pressures such as rapid climatic change will impact many threatened plants, and by extension this unique evolutionary wealth, is a major challenge we must arise to, requiring the development of new strategies and ways of thinking about conservation.

The study of plant secondary metabolites charts a long history in chemical ecology (Berenbaum 2014). In comparison to the now well-established field of plant-herbivore interactions, however, the ecological function and evolutionary significance of nectar secondary metabolites has only just recently begun to be appreciated (Adler 2000, Agrawal 2014). The power of mutualists to shape chemical defence in plants, via their interactions with nectar, thus represents a relative frontier in chemical ecology. The studies contained in this thesis may go at least some way towards placing pollinating mutualists in a deservedly more central role in the ecology of plant chemical defence. While defence against antagonists may indeed often take precedence in plants, it is now at least clear that, at least sometimes, mutualisms win the day.

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Appendices

Appendix 1. Populations and plants sampled for GTX analyses. Populations with asterisks are those which were included within studies in Chapter 3.

Range	Region	Population name	Code	Location	Elevation	No. plants sampled
Native	Spain	El Palancar Spring*	S1	36.081 N -5.543 W	495 m	8
		Llanos del Juncal*	S2	36.105 N -5.540 W	747m	8
		Above Calle Peña Prieta	S3	36.085 N -5.515 W	493 m	10
		Arroyo de Valdeinfierno	S4	36.227 N -5.611 W	150 m	8
		Garganta de Puerto Oscuro*	S5	36.518 N -5.632 W	605 m	9
		Garganta del Aljibe*	S6	36.538 N -5.635 W	469 m	7
	Portugal South	Foia*	PS1	37.316 N -8.589 W	861 m	7
		Monchique*	PS2	37.315 N -8.602 W	820 m	7
	Portugal North	Campia*	PN1	40.673 N -8.203 W	494 m	7
Invasive	Ireland	Crossover*	I1	52.894 N -6.400 W	165 m	7
		Dunran*	I2	53.060 N -6.102 W	156 m	8
		Tropperstown*	I3	53.017 N -6.274 W	185 m	8
		Shankhill*	I4	53.192 N -6.427 W	281 m	6
		Laois	I5	53.101 N -7.130 W	76 m	7
		Killarney	I6	52.005 N -9.513 W	32 m	7
		Recess	I7	53.467 N -9.737 W	13 m	7

Appendix 2

Temporal variation across floral phases

For investigation within season, seven plants were sampled from a native population (S1; Appendix 1) to examine the effect of floral development (or phenological phase) on nectar toxin levels. Two distinct nectar-producing stages of anthesis as defined by Mejías *et al.* (2002) were selected for comparison; namely α-phase (corolla newly opened i.e. 'young' flowers) and β-phase (pollen exposed; start of stigma receptivity i.e. 'mature' flowers). Mature flowers exhibited on average twice the level of toxin of young flowers – this difference being significant (Paired t-test, T= -7.05, p= 0.001). However, regression analysis revealed a strong linear relationship between the two phases (r^2 = 0.60, p= 0.044), indicating that rate of accumulation most likely follows a slow constant build-up over the lifetime of individual flowers.

Appendix 3.

Supplementary methods: data preparation preceding selection analyses

Analyses of covariance (ANCOVAs) were run to affirm the legitimacy of pooling samples between populations. For this, relative fitness was regressed on trait toxin content within each range, while also including a 'trait X population' interaction term. No significant interactions were found within either range (at an α-level of 0.05); indicating that samples could be satisfactorily pooled without masking important inter-population patterns.

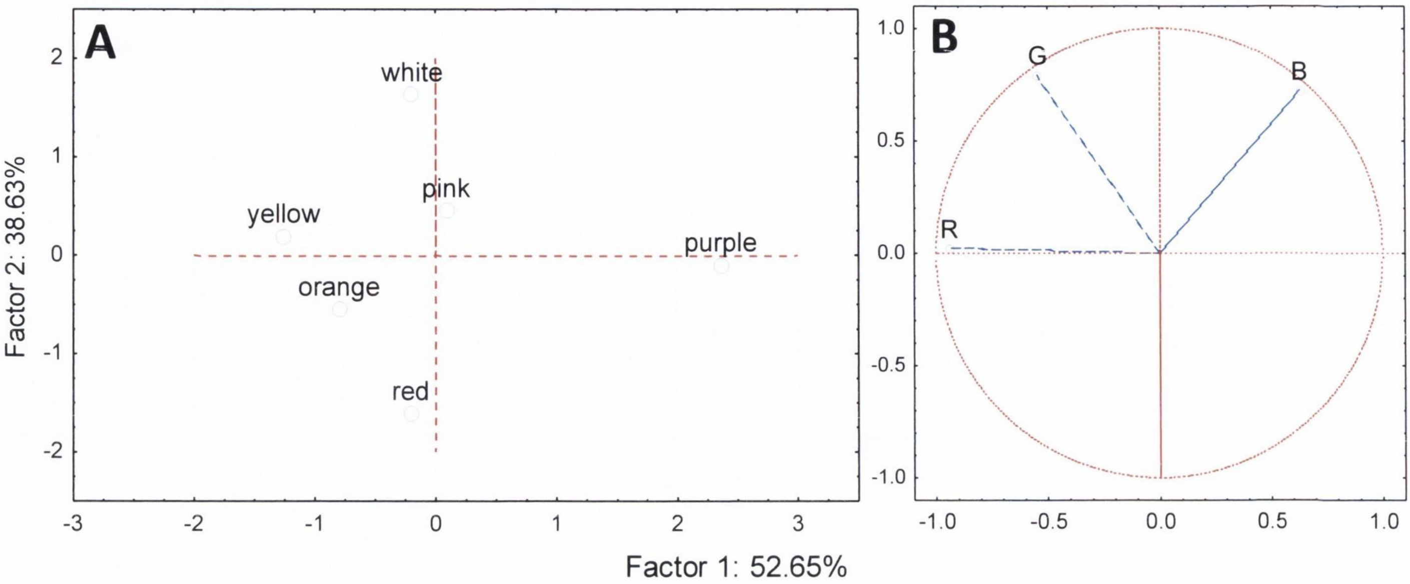
We previously revealed that several microhabitat variables (canopy cover, aspect, elevation and irradiance) explained a significant amount of variation in nectar toxin levels in *R. ponticum* (Chapter 2). To limit their potential contribution to the covariance between traits and fitness, these variables were combined into a single PCA component variable for the invasive and native range (accounting for 37 and 43 % of overall abiotic variation respectively), and regressed with toxin content for each tissue type. We took the residuals of these regressions as toxin content controlled for the potential influence of abiotic environment. We choose this 'partioning-out' approach over directly including the PCA variable within regressions, as the latter would bias estimates of goodness-of-fit for path models.

Appendix 4. Taxa of *Rhododendron* sampled in this study. Information is included on species' growth form (whether they are a shrub (S), tree (T), or both (S-T)); habit (whether epiphytic (E) or terrestrial (T), or both (E-T)); and elevational range. For species possessing multiple subspecific taxa, the default subspecies/variety was sampled unless otherwise indicated here. The ten species sampled across sections Pentanthera, Sciadorhodion, Tsutsusi were screened for presence/absence of nectar GTX, but were not included in further analyses due to low replication.

Subgenus	**Section**	**Species**	**Growth form**	**Habit**	**Elevation (m)**
Rhododendron	Vireya	*R. loranthiflorum*	S	E	180-1500
Rhododendron	Vireya	*R. herzogii*	S	E-T	1530-2500
Rhododendron	Vireya	*R. majus*	S	E-T	2770-3000
Rhododendron	Vireya	*R. gardenia*	S	E-T	1400-2300
Rhododendron	Vireya	*R. lochiae*	S-T	T	1200-1520
Rhododendron	Vireya	*R. luraluense*	S-T	E-T	900-1500
Rhododendron	Vireya	*R. gracilentum*	S	E-T	2000-2745
Rhododendron	Vireya	*R. armitii*	S	T	2250-2710
Rhododendron	Vireya	*R. anagalliflorum*	S	E-T	1190-3500
Rhododendron	Vireya	*R. apoanum*	S	E	1380-2930
Rhododendron	Vireya	*R. jasminiflorum*	S	E-T	1100-1500
Rhododendron	Vireya	*R. burtti*	S	E	1500-1600
Rhododendron	Vireya	*R. rarilepidotum*	S	T	1000-2500
Rhododendron	Vireya	*R. sarcodes*	S	E-T	900-2135
Rhododendron	Vireya	*R. ruttenii*	S	T	1900-2500
Rhododendron	Vireya	*R. taxifolium*	S	E	2600-2700
Rhododendron	Vireya	*R. polyanthemum*	S-T	E-T	1300-2300
Rhododendron	Vireya	*R. scarlatinum*	S	E	2950-3075
Rhododendron	Vireya	*R. suaveolens*	S-T	E-T	1200-1700
Rhododendron	Vireya	*R. lambianum*	S-T	E-T	1400-1600
Rhododendron	Vireya	*R. niveoflorum*	S	E-T	1300-2100
Rhododendron	Vireya	*R. praetervisum*	S	E	1100-1800
Rhododendron	Vireya	*R. verticillatum*	S	E	700-1500
Rhododendron	Vireya	*R. celebicum*	S	E	1800-2600
Rhododendron	Vireya	*R. retusum*	S-T	T	1350-3400
Rhododendron	Vireya	*R. vaccinioides*	S	E	1800-4200
Rhododendron	Rhododendron	*R. keysii*	S	T	2440-3650
Rhododendron	Rhododendron	*R. cinnabarinum*	S	T	2750-3950
Rhododendron	Rhododendron	*R. edgeworthii*	S	T	2100-3300
Rhododendron	Rhododendron	*R. genestierianum*	S	T	2450-4250
Rhododendron	Rhododendron	*R. glaucophyllum*	S	T	3050-3350
Rhododendron	Rhododendron	*R. pruniflorum*	S	T	3050-3950
Rhododendron	Rhododendron	*R. rubiginosum*	S-T	T	2500-3500
Rhododendron	Rhododendron	*R. russatum*	S	T	3400-4300
Rhododendron	Rhododendron	*R. dendricola*	S	E-T	1200-1400
Rhododendron	Rhododendron	*R. johnstoneanum*	S	T	1850-3100
Rhododendron	Rhododendron	*R. ciliatum*	S	T	2400-4000
Rhododendron	Rhododendron	*R. lindleyi*	S	E	2000-2750
Rhododendron	Rhododendron	*R. burmanicum*	S	T	2700-2900

Rhododendron	Rhododendron	*R. formosum var. inaequali*	S	T	1450-2230
Rhododendron	Rhododendron	*R. maddenii*	S	E-T	1900-2600
Rhododendron	Rhododendron	*R. scopulorum*	S	T	1950-2450
Rhododendron	Rhododendron	*R. saluenense ssp. chameunum*	S	T	3500-4500
Rhododendron	Rhododendron	*R. spinuliferum*	S	T	1800-2500
Rhododendron	Rhododendron	*R. oreotrephes*	S-T	T	2750-4250
Rhododendron	Rhododendron	*R. ambiguum*	S	T	2600-4500
Rhododendron	Rhododendron	*R. augustinii*	S	T	1300-3000
Rhododendron	Rhododendron	*R. siderophyilum*	S	T	840-2100
Rhododendron	Rhododendron	*R. yunnanense*	S	T	2100-3950
Rhododendron	Rhododendron	*R. zaleucum*	S	T	1800-3000
Hymenanthes	Pontica	*R. arboreum*	T	T	1850-3200
Hymenanthes	Pontica	*R. delavayi*	S	T	1500-3000
Hymenanthes	Pontica	*R. niveum*	T	T	2900-3650
Hymenanthes	Pontica	*R. argyrophyllum ssp. hypoglaucum*	S-T	T	1600-3650
Hymenanthes	Pontica	*R. wallichii*	S	T	3000-4000
Hymenanthes	Pontica	*R. callimorphum*	S	T	3000-4000
Hymenanthes	Pontica	*R. campylocarpum*	S	T	3000-4600
Hymenanthes	Pontica	*R. wardii*	S-T	T	3000-4300
Hymenanthes	Pontica	*R. souliei*	S	T	4300-4500
Hymenanthes	Pontica	*R. falconeri*	T	T	2700-3750
Hymenanthes	Pontica	*R. sinofalconeri*	S	T	2700-3750
Hymenanthes	Pontica	*R. rex*	S-T	T	3200-3500
Hymenanthes	Pontica	*R. coriaceum*	S-T	T	3000-4000
Hymenanthes	Pontica	*R. galactimim*	S-T	T	2900–3500
Hymenanthes	Pontica	*R. rothschildii*	S-T	T	3700-4000
Hymenanthes	Pontica	*R. semnoides*	S	T	3700-4000
Hymenanthes	Pontica	*R. hodgsonii*	S-T	T	3000-4000
Hymenanthes	Pontica	*R. vernicosum*	S-T	T	2600-3650
Hymenanthes	Pontica	*R. fortunei ssp. discolor*	S-T	T	600-2300
Hymenanthes	Pontica	*R. oreodoxa var. fargesi*	S-T	T	2650-4150
Hymenanthes	Pontica	*R. uvarifolium*	S-T	T	2100-4000
Hymenanthes	Pontica	*R. adenosum*	S	T	3350-3550
Hymenanthes	Pontica	*R. crinigerum*	S-T	T	3350-4000
Hymenanthes	Pontica	*R. habrotrichum*	S	T	2700-3350
Hymenanthes	Pontica	*R. sinogrande*	T	T	2450-4250
Hymenanthes	Pontica	*R. macabeanum*	T	T	2500-3000
Hymenanthes	Pontica	*R. grande*	T	T	2500-3000
Hymenanthes	Pontica	*R. montroseanum*	S-T	T	2500-3700
Hymenanthes	Pontica	*R. griersonianum*	S	T	2150-2700
Hymenanthes	Pontica	*R. irroratum ssp. ningyuenense*	S-T	T	2500-3500
Hymenanthes	Pontica	*R. irroratum ssp. pogonstylum*	S-T	T	2100-3000
Hymenanthes	Pontica	*R. longesquamatum*	S	T	2300-3350
Hymenanthes	Pontica	*R. morii*	S-T	T	2000-2200
Hymenanthes	Pontica	*R. pseudochrysanthum*	S	T	3800-4000
Hymenanthes	Pontica	*R. sperabile var. weihsiencse*	S	T	3000-3650
Hymenanthes	Pontica	*R. pocophorum var. hemidartum*	S	T	3650-4600

Hymenanthes	Pontica	*R. neriiflorum*	S-T	T	2750-3350
Hymenanthes	Pontica	*R. catacosmum*	S	T	3650-4400
Hymenanthes	Pontica	*R. dichroanthum*	S	T	2750-4550
Hymenanthes	Pontica	*R. haematodes ssp. chaetomallum*	S	T	3350-4450
Hymenanthes	Pontica	*R. microgynum*	S	T	3650-4250
Hymenanthes	Pontica	*R. sanguineum*	S	T	3000-4500
Hymenanthes	Pontica	*R. facetum*	S-T	T	2700-3350
Hymenanthes	Pontica	*R. yakushimanum*	S	T	500-2000
Hymenanthes	Pontica	*R. caucasicum*	S	T	500-2300
Hymenanthes	Pontica	*R. ponticum*	S	T	0-1800
Hymenanthes	Pontica	*R. degronianum ssp. heptamerum*	S	T	500-2000
Hymenanthes	Pontica	*R. hyperythrum*	S	T	900-1200
Hymenanthes	Pontica	*R. catawbiense*	S	T	0-800
Hymenanthes	Pontica	*R. selense ssp. jucundum*	S-T	T	3200-4100
Hymenanthes	Pontica	*R. faberi ssp. pratii*	S	T	3050-4400
Hymenanthes	Pontica	*R. wasonii*	S	T	2300-3000
Hymenanthes	Pontica	*R. cerasinum*	S	T	3200-3800
Hymenanthes	Pontica	*R. thomsonii*	S-T	T	3000-4000
Hymenanthes	Pentanthera	*R. luteum*	S-T	T	0-2300
Hymenanthes	Pentanthera	*R. occidentale*	S-T	T	0-2700
Hymenanthes	Pentanthera	*R. arborescens*	S-T	T	300-1500
Hymenanthes	Pentanthera	*R. calendulaceum*	S-T	T	180-1000
Hymenanthes	Pentanthera	*R. viscosum*	S-T	T	0-1500
Hymenanthes	Pentanthera	*R. canadense*	S	T	0-1900
Azaleastrum	Sciadorhodion	*R. schlippenbachii*	S-T	T	400-1500
Azaleastrum	Tsutsusi	*R. wadamim*	S-T	T	950-1500
Azaleastrum	Tsutsusi	*R. oldhamii*	S	T	0-2450
Azaleastrum	Tsutsusi	*R. rubropilosum*	S	T	2400-3000



Appendix 5. Principal component analysis of the predominant colours of *Rhododendron* flowers based on their combination of red, green and blue (RGB) values. The cumulative percent of variation represented from both axes was 91.3 %. Many species possess white flowers or white-flowering variants, however. That white flowers were quantitatively closer to pink flowers according to this ordination was indeed validated from our observations; in that if white and one or more other colour was listed for species' flower colour, 88 % of the time this would also include pink.

Appendix 6. Invasive alien species (IAS) which are threatened in their native range. A potential list of 25-30 species loosely fitting these criteria were narrowed down to the 12 presented here (10 animal, 2 plant species), based on combination of their native conservation status and reports of their intensity of invasion (which included a naturalised and/or instance colonization status across at least some parts of their introduced range).

Species	Type	Native range	Global / regional conservation status	Invasive range	References
Rhododendron (*Rhododendron ponticum*)	plant	Spain, Portugal	Portugal, EN – B1ac(ii,iii), Spain VU A1a; B2ab(i,ii,iii,iv)	Ireland, UK, NW Europe, New Zealand	(Griffin 1994, Mejías et al. 2002, Erfmeier and Bruelheide 2004)
Carp (*Cyprinus carpio spp.*)	animal	Afghanistan; Armenia (Armenia); Austria; Azerbaijan; Bulgaria; China; Croatia; Georgia; Germany; Hungary; Iran, Islamic Republic of; Kazakhstan; Kyrgyzstan; Moldova; Pakistan; Romania; Russian Federation; Serbia (Serbia); Slovakia; Tajikistan; Turkey; Turkmenistan; Ukraine; Uzbekistan	Vulnerable / Croatia EM France (mainland and Corsica) LC	Most continents and some 59 countries.	(Holčik 1996, Koehn 2004, Zambrano et al. 2006)
Monterey pine (*Pinus radiata*)	plant	USA (California) and Mexico	Endangered	South America, South Africa and other southern hemisphere regions. Cultivated in Australia, Chile, New Zealand	(Aljos et al. 1993, Berry et al. 2002, Rogers et al. 2006)
Barbary sheep (*Ammotragus lervia*)	animal	Northern Africa in Algeria, Tunisia, northern Chad, Egypt, Libya, northern Mali, Mauritania, Morocco, Niger and Suda	Vulnerable	SE Spain, SW United States, Niihau Island (Hawaii), Mexico, and some parts of Africa.	(Cassinello 1998, Acevedo et al. 2007)
Blackbuck antelope (*Antilope cervicapra*)	animal	India	Near Threatened	Argentina; United States, Australia	(Neginhal 1980, Raman et al. 1993, Merino et al. 2009)
Red-masked Parakeet (*Aratinga erythrogenys*)	animal	Ecuador and Peru	Near Threatened	Valencia (Spain) and in some Californian cities	(Best et al. 1995, Forys and Allen 1999, Schlaepfer et al. 2011)
European rabbit (*Oryctolagus cuniculus*)	animal	Europe	Near Threatened / France (mainland and Corsica) NT Republic of Ireland, Northern Ireland LC	All continents except Asia and Antarctica.	(Virgós et al. 2007, Lees and Bell 2008)
Java sparrow (*Padda oryzivora*)	animal	Indonesia (Java and Bali), and possibly also Madura	Vulnerable A2bde+3bde+4bde	Indian subcontinent, Colombo, Sri Lanka. In the United States there a breeding population on	(MacKinnon 2002, Kurniandaru 2008)

				several of the Hawaiian Islands	
Burmese python (*Python molurus bivittatus*)	animal	Asia	Vulnerable A2acd	USA	(Shah and Tiwari 2004, Engeman et al. 2011)
Chinese soft-shelled turtle (*Pelodiscus sinensis*)	animal	China (including Manchuria), Taiwan, North Vietnam, Korea, Japan and Russia	Vulnerable A1d+2d / Japan DD	Philippines	(Cheung and Dudgeon 2006, Chen and Lue 2010, Masin et al. 2014)
Javan rusa (*Rusa timorensis*)	animal	Java and Bali, Indonesia	Vulnerable C1	New Caledonia, Réunion	(Rouys and Theuerkauf 2003)
Sambar deer (*Rusa unicolor*)	animal	Indian Subcontinent and Southeast Asia	Vulnerable A2cd+3cd+4cd	Australia	(Timmins et al. 2011)

Appendix 7. The 24 climate change layers utilised in this study, based on climate change scenario, time periods and the ensemble model technique employed.

Combination no.	GCM	RCM	Scenario	Time period	Ensemble model technique
1	ECHAM4	RCA 30	A1b	2021-2050	Mean
2					Weighed mean
3					Median
4					Comm. averaging
5				2051-2080	Mean
6					Weighed mean
7					Median
8					Comm. averaging
9			A2	2021-2050	Mean
10					Weighed mean
11					Median
12					Comm. averaging
13				2051-2080	Mean
14					Weighed mean
15					Median
16					Comm. averaging
17			B1	2021-2050	Mean
18					Weighed mean
19					Median
20					Comm. averaging
21				2051-2080	Mean
22					Weighed mean
23					Median
24					Comm. averaging

Appendix 8. Climate variables and codes used. Variables in bold were used to build the models in the current study.

Code	Climate Variable
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	**Max Temperature of Warmest Month**
BIO6	Min Temperature of Coldest Month
BIO7	**Temperature Annual Range (BIO5-BIO6)**
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	**Mean Temperature of Coldest Quarter**
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	**Precipitation of Wettest Quarter**
BIO17	**Precipitation of Driest Quarter**
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter
ETP (PET)	**Potential Evapotranspiration (annual mean)**