DOCTORAL THESIS

Understanding the ecological and evolutionary consequences for plants following pollinator change

Christopher R. Mackin

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DECLARATION

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another university for the award of any other degree.

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Christopher R. Mackin

29th November 2021

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This thesis is a product of my own work and I have led the conception, data collection, analysis and writing of all chapters. The text conforms to a "papers style" format, in which chapters 2 – 6 take the form of discrete articles written in a style appropriate for publication in peer reviewed journals. Chapters 1 and 7 provide a general overview and discussion of the field and research undertaken.

Chapter 2 was submitted to and published with Journal of Ecology as "**Rapid evolution of a floral trait following acquisition of novel pollinators**" (Authors: Christopher R. Mackin, Julián F. Peña, Mario A. Blanco, Nicholas J. Balfour, Maria Clara Castellanos). Author contributions are as follows:

- CRM and MCC performed the field data collection with help from JFP, MJB and NAB. CRM processed the samples. MCC conceived the research. CRM and MCC performed the statistical analyses and wrote the manuscript. All authors critically commented on the manuscript and gave final approval for publication.

Chapter 3 is written in a style appropriate for submission to an ecological journal (Authors: Christopher R. Mackin, Maria Clara Castellanos). Author contributions are as follows:

- CRM and MCC conceived the research and methodology, CRM & MCC performed the data collection; CRM and MCC performed the statistical analyses and wrote the manuscript.

Chapter 4 was submitted to and published with Ecology & Evolution as "**Novel nectar robbing negatively affects reproduction in *Digitalis purpurea***" (Authors: Christopher R. Mackin, Dave Goulson, Maria Clara Castellanos). Author contributions are as follows:

- CRM and MCC conceived the research, CRM, MCC and DG conceptualised the experimental design and methodology, CRM performed the data collection, CRM and MCC performed the statistical analyses and wrote the manuscript. All authors critically commented on the manuscript and gave final approval for publication.

Chapter 5 is written in a style appropriate for submission to an ecological journal (Authors: Christopher R. Mackin, Alicia Sérsic, Valeria Paiaro, Eduardo Andrés Issaly, María Macarena García, Jeff Ollerton, Maria Clara Castellanos). Author contributions are as follows:

- CRM and MCC conceived the research, CRM, MCC and JO conceptualised the experimental design and methodology, CRM & MCC performed the data collection, AS, VP, AI and MMG provided resources critical for data collection. CRM and MCC performed the statistical analyses and wrote the manuscript with comment from JO.

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- CRM and MCC conceived the research, CRM, MCC and JO conceptualised the experimental design and methodology, CRM & MCC performed the data collection, AS, VP, AI and MMG provided resources critical for data collection. CRM and MCC performed the statistical analyses and wrote the manuscript.

University of Sussex

School of Life Sciences

Division of Evolution, Behaviour and Environment

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Abstract

Pollinators are important drivers for angiosperm evolution and diversification, but it is not well understood how changes in the pollinator assemblage can affect plant evolution in the short term. To address this question, my thesis investigates the consequences for plants of the addition and removal of floral visitors for two flowering species, _Digitalis purpurea_ and _Nicotiana glauca_, both of which recently colonised new continents and show variation in pollinators between their native and introduced ranges. In _D. purpurea,_ I found that populations in the introduced range pollinated by a new functional group (hummingbirds) had larger proximal corolla tubes than in the native range. Together with directional selection observed for the trait

in the introduced range, I demonstrate rapid evolution of proximal corolla tube traits following range expansion. Floral traits in this species show high heritabilities in all populations, in line with the ability to evolve rapidly. I also demonstrate that the addition of nectar robbers to *D. purpurea* plants, which are present in the introduced populations only, can cause significant negative effects on the female component of reproduction. In *N. glauca*, a plant pollinated by hummingbirds in the native range but self-pollinated in the introduced range, I found a relationship between stigma-anther distance and the rate of self-pollination in flowers but not at the level of the plant. I observed a high degree of within-individual variability for stigma-anther distances which may limit the response to selection for shorter stigma-anther distances to increase the rate of selfing. In this thesis, I show that the addition and removal of pollinators to the floral visitor assemblage of a plant can have consequences for floral trait evolution.

Dedicated to the memory of John Doughty Robson and Heather Robson

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Chapter 1

General Introduction

1.1 Introduction

Research on how plants evolve or change in response to a changing environment can uncover how organisms evolve in general, as well as contributing to efforts to understand and conserve biodiversity, ecosystem functioning and services (Frankel et al., 1995; Thompson, 2020). Studying the consequences of contractions and expansions of ranges of both plants and pollinators should involve examining plant populations undergoing changes in the pollination regime (González-Varo et al., 2013; Goulson et al., 2015), since this can have implications for the plant evolution and persistence. This introductory chapter will serve as a primer to my thesis, building up the background to the topic of floral evolution following pollinator regime change and highlighting the important but understudied areas in plant evolution research that my thesis contributes to.

Many flowering plants can persist and thrive under new environmental conditions through rapid adaptation (Salamin et al., 2010) and through flexibility in the breeding system (Peterson & Kay, 2015) among other ways. For instance, species may invade or colonise new areas where efficient and reliable pollination services they once experienced are not present. Colonisation events can involve changes in the mating system, with (for example) many self-compatible plants self-pollinating (selfing) either until pollinators co-opt the plant into their visitation networks (Issaly et al., 2020), or indefinitely following an evolutionary shift to selfing as a primary means of reproduction (Bodbyl Roels & Kelly, 2011). If novel pollinators are present in the new range but are functionally different to the plant's historic pollinator taxa, then there is potential for evolution of floral form to better accommodate the functional traits (such as mouthpart length and body size) of the novel pollinators. Although there is evidence from wild populations that there can be natural selection for novel floral form (Murúa et al., 2010; Temeles & Bishop, 2019), it has not yet been demonstrated that floral morphology can rapidly evolve following pollinator regime change. In addition to understanding the evolutionary consequences of the addition of novel pollinator mutualists to the visitor assemblage, we are also less certain of the effects of adding non-mutualists to the floral visitor assemblage. Here we distinguish mutualists as two interacting partners that both provide and receive a net benefit, whereas non-mutualists do not have a mutually positive interaction. Nectar robbers, for instance, have the potential to incur costs to plant reproduction through increased nectar production (Adler et al., 2016; Irwin et al., 2010) and reducing the attractiveness of the floral display (Navarro, 2001; Pyke, 1991) but scant evidence for negative effects of robbing exists (Stout et al., 2000). Research conducted as part of my thesis will endeavour to address these questions in pollinator regime change. In the

remainder of the introduction, I will focus on studies in evolution that cover aspects relevant to the study of the evolutionary and ecological consequences for plants of pollinator change.

1.2 Plant breeding systems and pollination

Hermaphroditic flowering plants typically contain within them male and female gametes on sexually separated reproductive parts (Barrett, 1998), which can be separated in space (spatial separation) and/or become receptive at different times (temporal separation) (Liedl & Anderson, 1993; Lloyd & Webb, 1986). Self-pollination can occur when pollen from anthers is transferred to stigmas of the same individuals (autogamy), either within the same flower with reduced stigma-anther separation (herkogamy) or pollen is transferred between flowers (geitonogamy; Opedal, 2018). Selfing holds many advantages for reproduction, especially when plants colonise new habitats, such as allowing plants to persist in environments lacking effective and frequent animal pollinators (Kalisz & Vogler, 2003). Also if a given genotype is well suited to a population, self-pollination increases the likelihood that an advantageous trait persists in a population (Barrett & Schluter, 2008). Selfing can also involve several disadvantages, including the lack of variation which leaves little potential for adaptations to arise to cope with a change in the environment, such as a new pathogen. Additionally, inbreeding depression can arise from selfing, meaning deleterious recessive alleles are more likely to be expressed (Goodwillie & Webber, 2018; Jarne & Charlesworth, 1993). Although exclusively selfing can be a successful strategy, many plants rely on a mix of reproduction by selfing and outcrossing by animal pollination.

Animal pollination has coevolved multiple times and has proved highly successful for a strategy to ensure reliable and efficient pollination by outcrossing (Herrera & Pellmyr, 2009). The transfer of different genes between individuals and populations is highly beneficial, as is the increased gene dispersal resulting from animals travelling large distances between pollination events (Campbell, 1985; Hartfield, 2016). However, much of the pollen produced is often wasted from the inefficiency of pollen transfer caused by the grooming behaviour of many pollinators (Castellanos et al., 2003) although presumably less pollen is wasted as compared with wind-assisted plant pollination. In addition, many pollinator taxa possess specialised morphology that requires compatible floral morphology, attractive signals and rewards, pollinator groups are often associated with particular floral traits (e.g. flower size, flower shape and colour). Pollination syndromes are a statistical construct where specific traits are overrepresented by plants pollinated by particular functional groups, and can be used as a tool to predict the identity

and characteristics of the pollinators from knowledge of the suites of floral traits possessed by the flower species (and vice versa) (Fenster et al., 2004). For instance, a commonly observed group of floral characteristics grouped in the bee 'syndrome' tend to be bilaterally (zygomorphic) or radially (actinomorphic) symmetrical, with yellow or blue flowers that offer sucrose-dominated nectar, that can have ultraviolet nectar guides (Fenster et al., 2004). However, these rules are often considered to have many exceptions, such as "hummingbird-syndrome" flowers (long, red corollas at a 45-degree angle) not always being visited solely by hummingbirds (Dellinger, 2020; Ollerton et al., 2009) and many hummingbird taxa visiting other types of flowers. Beyond expecting particular pollinating visitors from the floral morphology (and vice versa), many other visitors that are not exclusively beneficial for the plant can be expected to visit flowers, including herbivores and nectar robbers.

Nectar robbers collect nectar without contributing to pollination, either by piercing the corolla to feed (primary robbing) or taking advantage of existing holes (secondary robbing) (Inouye, 1983; Rojas-Nossa et al., 2016). Although many robber species capable of pollinating legitimately employ a mixed strategy of pollinating and robbing (Morris, 1996), some robbers (such as bumblebees) tend to stick to a single strategy throughout a foraging bout to reduce handling time (Bronstein et al., 2017). Collecting and depleting nectar rewards is thought to have a detrimental effect on the plant, potentially increasing metabolic costs through increased production of nectar and siphoning resources away from other critical processes such as seed production (Navarro, 2001; Pyke, 1991; Southwick, 1984). Additionally, both the depletion of nectar rewards and aesthetic damage to corollas can be off-putting to legitimate pollinators of the plant (Adler et al., 2016; Irwin et al., 2010). There is variation in the effects of nectar robbing reproductive success which could be dependent on context, with both evidence of no effects (Richman et al., 2018; Stout et al., 2000) and significant negative effects (Adler et al., 2016; Burkle et al., 2007; Castro et al., 2008; Irwin & Brody, 1999; Lara & Ornelas, 2001). More work is required to discover how nectar robbers affect plant reproduction.

Visitors to plants, where beneficial or antagonistic, can present new challenges to plants and alter selective pressures for plant traits. This can have implications for reproductive success and evolution.

1.3 Rapid evolution in plants and animals

Whilst major evolutionary changes over time such as speciation and development of whole taxonomic groups (macroevolution) can often be demonstrated, the occurrence of changes in

the frequency of a gene in a population over shorter periods of time (microevolution) is less frequently observed (Reznick & Ricklefs, 2009). In addition, field studies on plant populations often show weak natural selection on floral traits, suggesting limited potential for evolution. This contradiction between macro- and micro- evolutionary change could potentially be explained by long periods of stasis interspersed by short and occasional periods of exposure to new environmental conditions causing rapid evolution (Hairston Jr et al., 2005; Thompson, 1998). The detection of microevolution would therefore be increasingly likely if organisms were studied following a substantial change in their environment, such as a change in the functional group pollinating a flowering plant.

Rapid evolution has been demonstrated for many focal species for animals, such as in Darwin's Finches evolving in response to fluctuating rainfall (see Grant & Grant, 1992 and freshwater copepods in response to fluctuating fish predation (Hairston & Dillon, 1990) with both examples showing high rates of evolutionary change detectable over a short period (reviewed in Hairston Jr et al., 2005). There are also many examples of rapid evolution in plant traits, such as in size and fecundity (Maron et al., 2004) and life history traits (Franks & Weis, 2008). Arguably a major factor driving diversification of floral forms is through Darwin's coevolutionary race model, whereby a gradual increase in compatibility between functional traits of both the pollinating animal and the plant leads to evolutionary changes over time for both interacting partners (Whittall & Hodges, 2007). A unique opportunity to study rapid evolution could use recent plant colonists that have experienced a change in the pollinator mutualist that ensures its reproductive success, then observing any differences in traits and selection patterns between ranges.

1.4 Rapid floral evolution following pollinator changes

New pollinator environments can lead to novel selection pressures for new floral form in plants, especially for floral traits that improve access to rewards, the floral display attractiveness or mechanical fit between flower and pollinator (Newman et al., 2015; Van der Niet et al., 2014). Evidence for this is shown by both macroevolutionary patterns of adaptations (e.g. pollination syndromes; Fenster et al., 2004) where floral trait evolution is in response to selection imposed by traits of the species in the functional group (such as mouthparts) and through adaptive geographical variation that results from historic differences between pollinators in areas that lead to local floral ecotypes (e.g. Anderson et al., 2010; Herrera et al., 2006; Valiente-Banuet et al., 2004). In artificial selection experiments, novel pollinator environments have been

demonstrated to cause selection for a particular trait and lead to rapid evolution in just a few generations (Lehtilä & Holmén Bränn, 2007; Lendvai & Levin, 2003; Worley & Barrett, 2000). So in theory, changes in the pollinator assemblage could result in rapid floral trait evolution in natural populations. Work related to this has found that removal of pollinators can cause changes in the mating strategy of plants and associated reproductive traits (Issaly et al., 2020), such as evolution towards uniparental reproduction following absence of animal pollination, both via increased cloning (Castro et al., 2016; Ferrero et al., 2020) and increased selfing (Bodbyl Roels & Kelly, 2011; Petanidou et al., 2012; Ward et al., 2012). In the case of increased self-pollination, selection for increased reproduction by selfing can cause both an increase in the degree to which flowers are self-compatible alongside a reduction in the stigma-anther distances (Bodbyl Roels & Kelly, 2011). It is less clear how floral traits evolve or change in scenarios where the functional groups that pollinate the plant change, even when there are not necessarily changes to the breeding system.

Research demonstrating rapid evolutionary innovation in corolla traits in wild plants are rare, with one notable exception in a study by Campbell et al., (2018) who found contemporary evolution of *Ipomopsis* corolla length in approximately five generations. In addition to this, a few studies have demonstrated novel selection patterns following a change in the pollinator assemblage. Murúa et al., (2010) showed that a change in the visiting pollinator assemblage was associated with altered selection for novel corolla shape and flower number, when comparing flowers in native forest and human-disturbed forests. In addition to this, a study by Temeles & Bishop (2019) found that, following a hurricane in Dominica that displaced bird pollinators of *Heliconia wagneriana*, the new shorter-billed hummingbird pollinators exerted strong selection for shorter corollas. Both of the previous examples of novel selection on floral form following a change in the pollinator environment would require subsequent observational study to determine whether floral evolution has occurred over the period of change. However, no study has of yet investigated the evolutionary consequences for plants when an entirely new functional group of pollinators are added to the visiting assemblage. Rapid adaptation to visitation by a new floral visitor assemblage could be an important factor in the evolution of floral diversity in angiosperms (Harder & Johnson, 2009) and this question is also relevant in terms of global pollinator decline and changes in pollinator distributions due to climate change. Changes in the climatic envelope of pollinator species could lead to changes for some plant populations in the visiting functional taxa (Gómez-Ruiz & Lacher, 2019), and thus likely lead to alterations in selection pressures for plants (Gilman et al., 2012; Morton & Rafferty, 2017) .

Rapid evolution of floral traits could be further evidenced by comparing populations of a recent plant colonist that shows morphological differentiation that correlates with functional traits sizes of novel pollinators. Further work on such a system could test for natural selection (and the strength of selection) and quantify the heritability (proportion of underlying variation that is due to additive genetic variance), which would both allow an estimation of potential evolutionary change over time.

1.5 Study Systems

In order to study the evolutionary and ecological changes following changes in pollinator assemblage, I chose two focal plant species with recorded differences in their visiting pollinator taxa between populations of the same species. The herbaceous facultative biennial common foxglove, *Digitalis purpurea*, was chosen due to observational evidence that some populations in the introduced range are visited by hummingbirds, and multiple species of bumblebee (Riveros et al., 2006). For a study system that involved a change from hummingbird pollination to no animal pollination, I chose the long-lived woody tree tobacco, *Nicotiana glauca*, which is bird pollinated in some populations and relies on self-pollination in other populations (Ollerton et al., 2012). Thus, *N. glauca* is self-compatible and can therefore set seed without animal visitation, whilst *D. purpurea* is not self-incompatible but requires animal vectors to ensure full seed set. For animal visitation in both species, long tongues and a particular body size in bumblebees and a length of beak for birds are required in order to access the nectaries at the base of the flower (Schueller, 2004). Both species express toxins in the nectar and other organs of the plant in order to deter herbivores (Evans & Cowley, 1972, Furer et al., 2011) and both plants tend to germinate and grow in areas of disturbance (Grindeland et al., 2005; Nattero & Cocucci, 2007). Of utmost importance is the differences in plants in the changes in pollinator community between the ranges, allowing different research questions to be examined for each study system.

1.6 Aims and objectives

In my thesis, I aim to understand the outcomes for plants following changes in the pollinator assemblage, including additions of novel floral visitors and removal of animal pollination. The changes I investigate include; (i) additions of novel pollinator species and pollinator groups to the visitor assemblage; (ii) addition of nectar robbers to the visiting assemblage and (iii) removal

of animal pollination from the plant's environment. I examined two study systems in depth: *Digitalis purpurea* (the common foxglove) for parts (i) and (ii), and *Nicotiana glauca* (tree tobacco) for part (iii). I chose *D. purpurea* as a study system due to the plant being a well-known specialist on long-tongued bumblebees in the native range, with apparent hummingbird visitation in populations within the introduced range. I selected *N. glauca* as an additional focal plant based on literature suggesting differences in floral traits between populations that corresponds with their pollinator taxa or lack of animal pollination.

The first data chapter (chapter 2) compares morphology and selection patterns in native and introduced populations of *Digitalis purpurea* to ascertain if the addition of a new functional pollinator group, hummingbirds, has led to rapid evolution of corolla traits. Using seeds collected from native and introduced *D. purpurea* populations visited in chapter 2, chapter 3 utilises a common garden experiment to examine differences in floral trait means between ranges when grown in similar environmental conditions, to find how the trait expression is controlled. This chapter allows an estimation of the heritability of traits, demonstrating the proportion of the variation that due to additive genetic variance. We can also infer whether there are local adaptations of plants suited to their populations of origin. Chapter 4 uses experiments on potted plants of *D. purpurea* to simulate nectar robbing and determine if there are potentially any negative effects of robbing on introduced populations whose visitor assemblage now includes nectar robbers. Chapters 5 and 6 investigate a second study system, *Nicotiana glauca,* a hummingbird pollinated plant that has expanded its range into areas that include populations lacking animal pollinators. Chapter 5 investigates how selfing is controlled in this plant species, and tests for a relationship between the rate of reproduction by self-pollination in flowers and floral traits including the stigma-anther distance. Chapter 6 builds upon this by growing plants from native and introduced populations in a common garden experiment, in order to understand how variation in floral traits is determined at the population level and whether phenotypic plasticity plays a role in variation. This chapter includes a calculation of the heritability of floral traits (including SA distance) and an estimation of how plastic the traits can be. Finally, the conclusions arising from my findings from the data chapters are summarised and expanded upon in chapter 7. The results are discussed within a broader evolutionary context and areas for future research are suggested. I explore the implications of the findings of this thesis for plant floral evolutionary change.

Chapter 2

Floral trait evolution in *Digitalis purpurea* following acquisition of novel pollinating taxa

Abstract

Changes in the pollinator assemblage visiting a plant can have consequences for reproductive success and floral evolution. We studied a recent plant trans-continental range expansion to test whether the acquisition of new pollinator functional groups can lead to rapid adaptive evolution of flowers. In *Digitalis purpurea,* we compared flower visitors, floral traits and natural selection between native European populations and those in two Neotropical regions, naturalised after independent introductions. Bumblebees are the main pollinators in native populations, while both bumblebees and hummingbirds are important visitors in the new range. We confirmed that the birds are effective pollinators and deposit more pollen grains on stigmas than bumblebees. We found convergent changes in the two new regions towards larger proximal corolla tubes, a floral trait that restricts access to nectar to visitors with long mouthparts. There was strong positive linear selection for this trait in the introduced populations, particularly on the length of the proximal corolla tube, consistent with the addition of hummingbirds as pollinators. The addition of new pollinators is likely to happen often as humans influence the ranges of plants and pollinators, but it is also a common feature in the long-term evolution of the angiosperms. We show how novel selection followed by very rapid evolutionary change can be an important force behind the extraordinary diversity of flowers.

Key Words: bumblebee, contemporary evolution, *Digitalis purpurea*, floral evolution, hummingbird, pollinator change

2.1 Introduction

Changes in pollinator communities and plant range shifts are currently affecting pollination interactions across many ecosystems (González-Varo et al., 2013; Grass et al., 2014; Goulson et al., 2015) and, in consequence, many plants are experiencing new pollinator environments. From the plant's perspective, rapid changes in pollination interactions can have implications on crucial processes such as reproductive success and, eventually, their evolution. Even over very short time scales, new pollinator environments can potentially lead to novel selection pressures on mating strategies and floral traits. For instance, there is evidence of range changes favouring shifts to increased levels of uniparental reproduction if pollinators become scarce or are completely lost, both via increased cloning (Castro et al., 2016; Ferrero et al., 2020) or via shifts to self-pollination (Bodbyl Roels & Kelly, 2011; Petanidou et al., 2012; Ward et al., 2012). In the latter cases, the degree of self-compatibility or floral morphological traits that favour selfing, such as the distance between anthers and stigmas, show adaptive changes that provide reproductive assurance in the absence of pollinators. We are less certain about the implications for floral evolution in situations where the pollinator community changes to include new functional groups of floral visitors, which could select for new floral traits without necessarily changing the breeding system.

Over long timescales, pollinators are important agents of selection of floral morphological traits that increase the mechanical fit between flower and pollinator, regulate access to rewards, and optimize the attractiveness of the floral display. Evidence for this comes both from macroevolutionary patterns of adaptation to different pollinator functional groups (e.g. pollination syndromes, reviewed by Fenster et al., 2004) and adaptive intraspecific geographic variation resulting from historical local dissimilarity between pollinators, producing floral ecotypes (e.g. Valiente-Banuet et al., 2004; Herrera et al., 2006; Anderson et al., 2010, Paudel et al., 2016). Furthermore, artificial selection experiments consistently show that floral morphological traits can evolve in response to a changed pollination environment in a few generations (e.g. Worley & Barrett, 2000; Lendvai & Levin, 2003; Lehtilä & Holmén Bränn, 2007; Gervasi & Schiestl, 2017). In principle then, changes in the pollinator environment can be expected to lead to very rapid evolution of floral traits in wild populations as well. Much of the research addressing this question in the field has focused on potential changes in the mating strategies and reproductive morphology that rapidly occurs when invasive plants lose animal pollination altogether and resort to self-pollination (Issaly et al., 2020). However, to our knowledge no studies have investigated the short-term evolutionary consequences for plants of the addition of entirely new functional pollinator groups, as opposed to a reduction in pollinator

diversity. This is relevant not only in the context of global pollinator changes, but also because rapid adaptation to new pollinator environments could be a key driver of angiosperm floral diversity. Such adaptation is most likely to happen in response to selection for novel phenotypes as plants are exposed to new pollinators (Harder & Johnson, 2009), while not necessarily losing their previous ones.

A unique opportunity to address this question comes from recent plant range expansions into areas where they are exposed to novel pollinator taxa. In this study we use the short-lived and facultatively biennial herb *Digitalis purpurea* as a focal species to test if a change in pollinator assemblage after the recent colonization of a new continent leads to adaptive changes in floral morphology. In its native range in Western and Northern Europe, *D. purpurea* is pollinated by a few species of bumblebees (Grindeland et al., 2005; Broadbent & Bourke, 2012), but in naturalised populations in the Americas, hummingbirds have also become frequent floral visitors. With the addition of this new functional group of pollinators, our hypothesis is that the new pollinator environment will impose a different selection regime on flowers and lead to changes in floral traits, as for example, longer corollas typical of hummingbird-pollinated flowers. The convergent floral syndrome associated with hummingbird pollination across angiosperm families suggests that selection imposed by birds in particular can be strong (Caruso et al., 2019; Pauw, 2019), especially when there is poor morphological matching with the flower to begin with (Nattero et al., 2010). Alternatively, naturalised populations of *D. purpurea* could accommodate new pollinators without any detectable divergence in floral traits.

To test this, we focus on the comparison of native populations (from Southern England) with naturalised populations in two non-native areas where hummingbirds are reported as visitors (Colombia in South America and Costa Rica in Central America). We identified pollinators and quantified their visitation rates and pollen transfer effectiveness. In the same populations, we measured floral morphology and nectar characteristics, and quantified natural selection on these traits. Convergent variation in floral traits associated with new pollinators in independently evolving naturalised populations can provide evidence for rapid adaptation to novel pollination environments.

2.2 Materials and Methods

2.2.1 Study system and field sites

Digitalis purpurea L. (Plantaginaceae) is a facultatively biennial herb that depends on light gaps or disturbed sites for germination and establishment. Seeds persist in the seed bank and can form densely populated aggregations. *D. purpurea* is semelparous, with most individuals flowering only once in their lifetimes. On the second summer after germination (although this is sometimes delayed for one or more years), rosettes produce large showy inflorescences with several dozen flowers that open sequentially from the bottom to the top of the inflorescence. The purple flowers are bell-shaped and protandrous, with anthers dehiscing shortly after anthesis, while the stigma becomes receptive (by unfolding its two lobes) up to five days later (Darwin, 1876). The plant is self-compatible, but insect visitation is required for full seed set (Nazir et al., 2008; see also Results). Bumblebees typically fly upwards when foraging on an inflorescence, so on *D. purpurea* they travel from older female phase flowers lower in the inflorescence to male phase flowers higher up in the inflorescence, potentially reducing the incidence of self-pollination (Best & Bierzychudek, 1982). The main pollinator in the native European range is the garden bumblebee, *Bombus hortorum,* found to be the predominant visitor to plants in Britain and Norway (Manning, 1956; Grindeland et al., 2005; Broadbent & Bourke, 2012). The same studies report that other *Bombus* species with long tongues, such as *B. pascuorum*, can also be frequent visitors and pollinators of *D. purpurea*. Visitation by insects with shorter mouthparts is likely restricted by the narrow restriction of the corolla tube at its proximal part (Fig. 2.1).

Digitalis purpurea is native to Western Europe, including the British Isles, but has become naturalized in many temperate regions and tropical highlands of the world (Heywood, 1951; Bräuchler et al.*,* 2004). Populations in South and Central American mountains likely originate from garden escapees imported by English engineers (Calle et al., 1989; Díaz, 2011). Precise dates of the introductions are not available for either country, but no records of the plant are present in A.S. Ørsted´s (1863) thorough description of the flora of Costa Rica from 1846–1848. Pérez-Arbeláez (1978) cites a botanical collection in Colombia from 1856 where the plant is described as a recent introduction. The first herbarium records date from 1928 in Costa Rica (www.tropicos.org) and 1932 in Colombia (Virtual Herbarium, Universidad Nacional de Colombia). It is thus likely that the introductions happened sometime around the 1850´s. Because this is a biennial species, we can assume there have been <85 generations in the introduced areas. The two regions included in this study are separated by strong geographical barriers, including the vast lowland forests in the Panama isthmus where *D. purpurea* would not

survive, so that it is highly unlikely that the populations in Central and South America have a single origin with subsequent natural dispersal. Human-mediated dispersal from one region to the other would still be a possibility, but preliminary molecular results firmly suggests independent introductions. A dataset comprising ~9K single nucleotide polymorphisms (SNPs) confirm that British populations are ancestral to both naturalised regions, and that populations in Colombia, Costa Rica and Britain cluster together within each region (= country), with very low or no admixture with the other two regions. The two tropical regions are also strongly divergent in this multilocus analysis, and given their recent establishment, this further supports the fact they originated from independent introduction events (Castellanos et al., unpublished).

Figure 2.1. Longitudinal section of *Digitalis purpurea* flower with part of the corolla and one stamen removed. The floral nectaries are located at the base of the ovary, within the constricted proximal part of the corolla tube.

In the new range, plants can flower throughout the year (although plants within a population tend to be in synchrony for flowering) and hummingbird visitation is frequently observed (Castellanos, pers. obs.; Riveros et al., 2006). In Andean Colombia, the bumblebee species *Bombus hortulanus*, *B. atratus* and *B. rubicundus* have been reported to visit and rob *D. purpurea* flowers (Riveros et al., 2006).

Eleven populations of *D. purpurea* across the native and non-native range were chosen for comparisons of pollinator assemblage and floral morphology (Table 2.1), including four populations in Britain (Fig. A2.1), five in Colombia (Fig. A2.2) and two in Costa Rica (Fig. A2.3). In a subset of them, we measured nectar and vegetative traits, and performed experiments to detect potential changes in the breeding system and pollen limitation. In five of these populations we also measured natural selection on floral traits (Table 2.1). Fieldwork took place between 2016 and 2019.

2.2.2 Breeding System

We used controlled hand pollinations to study the breeding system and assess the potential pollen limitation in three of the study populations (two native, one introduced; Table 2.1). Four different pollination treatments were applied to individual flowers on the same individual plants, for 8-20 individuals per population. The treatments were: i) an emasculated flower manually outcrossed using fresh pollen from another plant ("manually outcrossed"), ii) an emasculated flower manually selfed using pollen from another flower on the same plant ("manually selfed"), iii) a non-pollinated flower with normal anthers to allow for autonomous selfing ("naturally selfed"), and iv) an open "control" flower. Flowers in treatments i) to iii) were covered with bridal veil bags while still in bud to prevent any pollinator visits. We removed bags after the flowers wilted to allow normal fruit development. Flowers in treatments i) and ii) were emasculated by removing undehisced anthers using tweezers while still in bud, and hand-pollinated a few days later when the stigmas became receptive. After 4–8 weeks we collected undehisced fruit capsules and left them to dry in separate paper envelopes. The seeds were extracted from fruits in the laboratory, photographed and counted using ImageJ 1.52e software (http://rsb.info.nih.gov/ij/). Seed counts were compared across treatments with linear models in R.

Table 2.1. *Digitalis purpurea* populations included in this study, with datasets collected. Coordinates are in WGS 84 degrees.

Region	Population	Coordinates (latitude, longitude)	Elevation (m)	Pollinator censuses	Floral Morphology	Nectar traits	Vegetative traits	Selection on traits	Breeding system
Britain (native)	Loder Valley	51.055, -0.093	98	✓	✓	✓	✓	✓	
	Holy Cross	50.972, 0.199	128	✓	✓	✓	✓	✓	✓
	Calcot Wood	50.921, -0.332	25	✓	✓				✓
	Ashdown Forest	51.089, 0.153	113		✓				
Colombia (non-native)	Choachí	4.592, -74.031	3270	✓	✓	✓	✓	✓	
	Floresta Reserve	4.802, -73.998	3050	✓	✓	✓	✓	✓	✓
	Guatavita	4.979, -73.773	3000		✓				
	La Vieja	4.711, -74.011	3025		✓				
	Encenillo	4.789, -73.909	3080		✓				
Costa Rica (non-native)	La Georgina	9.559, -83.724	3070	✓	✓	✓	✓	✓	
	Cuericí	9.555, -83.667	2565		✓		✓		

2.2.3 Characterising pollinator assemblages and quantifying visitation

We quantified pollinator activity when the populations were in full bloom by surveying *D. purpurea* plants during a series of three-minute censuses (June and July in Britain, December and February in Colombia, March and April in Costa Rica) covering all times of the day that floral visitors are active, including dawn in the tropical populations. Surveys took place over 4-9 days during a single flowering season between 2016 and 2019 for most populations, except for Calcot Wood and Holy Cross in Britain that were studied on two consecutive summers. For each census, we surveyed multiple inflorescences containing 20–50 flowers and recorded i) the number of flowers surveyed, ii) the species of visitor, and iii) the number of flowers visited in the three-minute period. Considering potential observer bias due to the shy nature of hummingbirds, recorders ensured distance from the inflorescences and with green or dark clothing to help maintain concealment. We then estimated legitimate visitation rates as the number of visits per flower per hour. These were compared between populations using generalised linear models followed by Tukey's pairwise comparisons using function 'glht' from the R package *multcomp* (Hothorn et al., 2008). Data is likely to fit a poisson distribution and be zero inflated due to count data.

For visiting bumblebee species in the non-native range with no published functional morphological measurements, we collected specimens and measured their tongue lengths (glossa plus prementum) for comparisons with pollinators in the native range.

2.2.4 Effectiveness of pollinators

As one measure of their pollination effectiveness, we compared the ability of common visitors at delivering pollen to virgin stigmas after a single visit in two native British populations, and three non-native populations. For this, we emasculated flowers while still in the bud stage and bagged them to prevent any visits. Once the stigma on a flower had become receptive a few days later, bags were removed and the plant monitored for visits from a pollinator. Immediately after a single legitimate visit, we identified the pollinator and squashed the flower´s stigma on a microscope slide using fuchsin-stained glycerine jelly. This was repeated for as many pollinator species as possible, and all conspecific pollen grains were counted under a microscope with help from photographs if needed. We tested for differences among functional groups of pollinators using analysis of variance and running paired Tukey tests in the 'base' package in R.

2.2.5 Comparisons of floral morphology and nectar traits

A minimum of 40 healthy plants were chosen haphazardly from each of the eleven populations (between 2015 and 2019) for morphological characterization. Between three and four flowers were collected from different positions in each inflorescence to account for any intra-plant variation in floral traits. Picked flowers were pressed in filter paper, dried in an oven at 45ºC for at least two days, and for a further one day immediately before measuring. After drying, flowers were weighed on a precision balance to the nearest 0.001g for a measure of dry weight.

We then used digital images of the pressed flowers to measure whole corolla length, whole corolla height, proximal corolla tube length and proximal corolla tube width, using ImageJ software (Fig. 2.2). Strictly speaking, we are measuring the *height* of the proximal corolla tube (see Fig. 2.2), but because this section of the corolla tube is roughly cylindrical, the width and the height are approximately the same. We refer to it as *width* for consistency with previous studies in corolla evolution. As expected, all four traits co-vary significantly to some extent within each population, with the strongest correlations occurring between whole corolla height and whole corolla length (pearson correlation coefficient up to $r = 0.71$), and proximal corolla length and proximal corolla width (up to $r = 0.51$). For the morphological comparison analysis we therefore use the geometric mean of the length and height of the whole corolla ("whole corolla size" hereafter) and the geometric mean of the length and width of the proximal corolla tube ("proximal corolla tube size" hereafter). We keep the whole corolla tube size and proximal corolla tube size as separate traits, because the proximal tube is the constricted part at the base of the corolla tube restricting access to the nectaries for floral visitors with short mouthparts (see Fig. 2.1), and thus we are interested in the functional role it may play in different pollinator environments. Two vegetative traits were also measured for all plants in each population: rosette diameter (taken as the longest linear measurement from leaf tip to leaf tip across the rosette), and inflorescence height to the first flower (i.e., the peduncle, equivalent to the height from the base of rosette to the first flower on the inflorescence).

Trait measures were compared between the native and non-native range using mixed-effects linear models with population and individual plant as random factors. Linear models were programmed using the 'lmer' function in the *lme4* R package (Bates et al., 2015).

We measured nectar traits in five of the study populations (Table 2.1). We bagged inflorescences and after 24 hours, we picked two to three flowers in the male phase from each plant and used microcapillary tubes to measure the volume of nectar from the base of the corolla. We then used a pocket refractometer to estimate the sugar concentration.
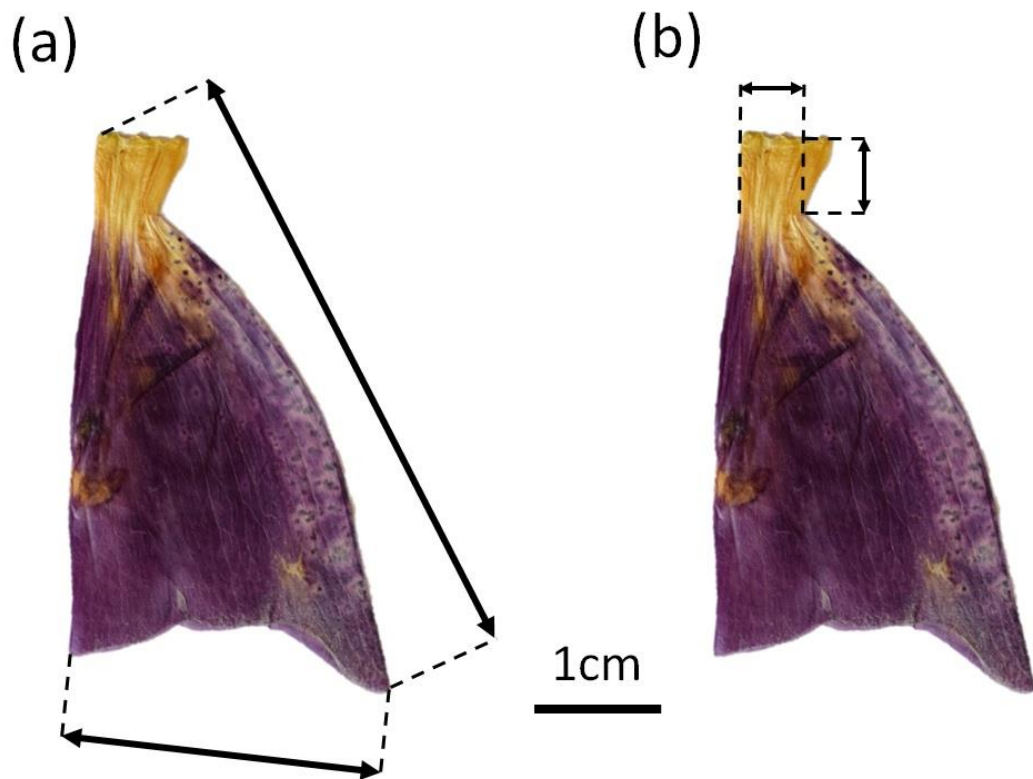
Figure 2.2. Pressed *Digitalis purpurea* flowers illustrating the morphological measurements taken. (a) Whole corolla length and height; (b) Proximal corolla tube length and width

2.2.6 Natural selection on floral traits

We estimated total seed production by all focal plants in five of the study populations (Table 2.1) as a proxy for lifetime female fitness. Three ripe but undehisced fruits were collected from each of three different positions in the inflorescence (lower, middle and upper). This was done to account for any intra-plant variation in resources allocated to fruits at different ages of the plant, as our previous observations suggested that fewer seeds may be produced by fruits later in the season. To obtain total seed production, we multiplied the average number of seeds produced by these three fruits by the number of successful fruits produced in the lifetime of an individual plant. We estimated the total number of fruits from the numbers of flowers, as the initial count of flowers correlates strongly with the number of fruits produced ($r^2 = 0.95$, $P < 0.001$, $N = 116$, in two populations in Britain).

We measured both linear and non-linear natural selection acting on floral traits in each population using lifetime seed production as a measure of female fitness. We estimated selection parameters using the general additive model (GAM) approach on absolute fitness values implemented by Morrissey and Sakrejda (2013). We report linear (β) and quadratic (γ) selection gradients on corolla traits estimated in bivariate models that included both whole corolla size and proximal corolla size, to control for correlations between the traits. Selection gradients estimate selection on each of the two traits, considering the other one simultaneously, and therefore estimate direct selection on each (Lande & Arnold, 1983). In addition, we ran separate univariate selection analyses for the four corolla tube traits in each population, to get further insight into the targets of selection. For nectar traits we also estimated selection gradients in bivariate models where both the volume of nectar and sugar concentration were included. We fitted GAM models using the *mgcv* package in R (Wood, 2011), and then calculated the statistical significance of selection coefficients via the bootstrap approach (N > 500 iterations) implemented in package *gsg* (Morrissey & Sakrejda, 2014). Standardisation of traits and fitness values, as well as scaling of quadratic coefficients (as explained in Stinchcombe et al., 2008) are automatically implemented by the *gsg* calculations.

2.3 Results

2.3.1 Breeding system

Results of hand pollinations in two native *Digitalis purpurea* populations (Holy Cross and Calcot Wood) were compared with one population in the introduced range (Floresta). Across all populations, hand pollination treatments produced significantly different numbers of seeds (estimate = 801.5; P < 0.001; N = 8 to 20 flowers per treatment in each population; Fig. 2.3). Post-hoc tests show that flowers bagged for autonomous selfing ("naturally selfed") produced significantly fewer seeds than the control both in all populations in Britain (P < 0.001) and Colombia (P < 0.001), often aborting and not producing any viable seeds. This is consistent with previous reports for this species in the native range (Darwin, 1876) and confirms that non-native populations are also dependent on pollinators for seed production.

The manually self-pollinated flowers produced similar numbers of seeds (mean = 909 ± 90 SE in Britain and 559 ± 103 in Colombia) to the control (849 ± 90 for Britain and 736 ± 102 in Colombia) and outcrossed treatments (787 ± 63 in Britain and 801 ± 75 in Colombia), and differences between these were not statistically significant in post-hoc tests (all with P < 0.001); this confirms full self-compatibility (Darwin, 1876; Fig 2.3). The number of seeds produced in the

manually outcrossed treatment and the control treatment was not significantly different in Colombia (P = 0.93) or Britain (P = 0.92), indicating no pollen limitation for this species in either the native or non-native range (Fig 2.3). We do not have a treatment for open flowers with extra pollen, although we do get full seed set in the above treatments.

Figure 2.3 further shows that seed production was variable within treatments in both ranges and post-hoc tests confirmed that mean seed production in each treatment was not significantly different across regions. Note that the spontaneous selfing treatment ("naturally selfed") showed more variance and higher mean values of seed set in the introduced population; however, this was not significantly different from the native populations.
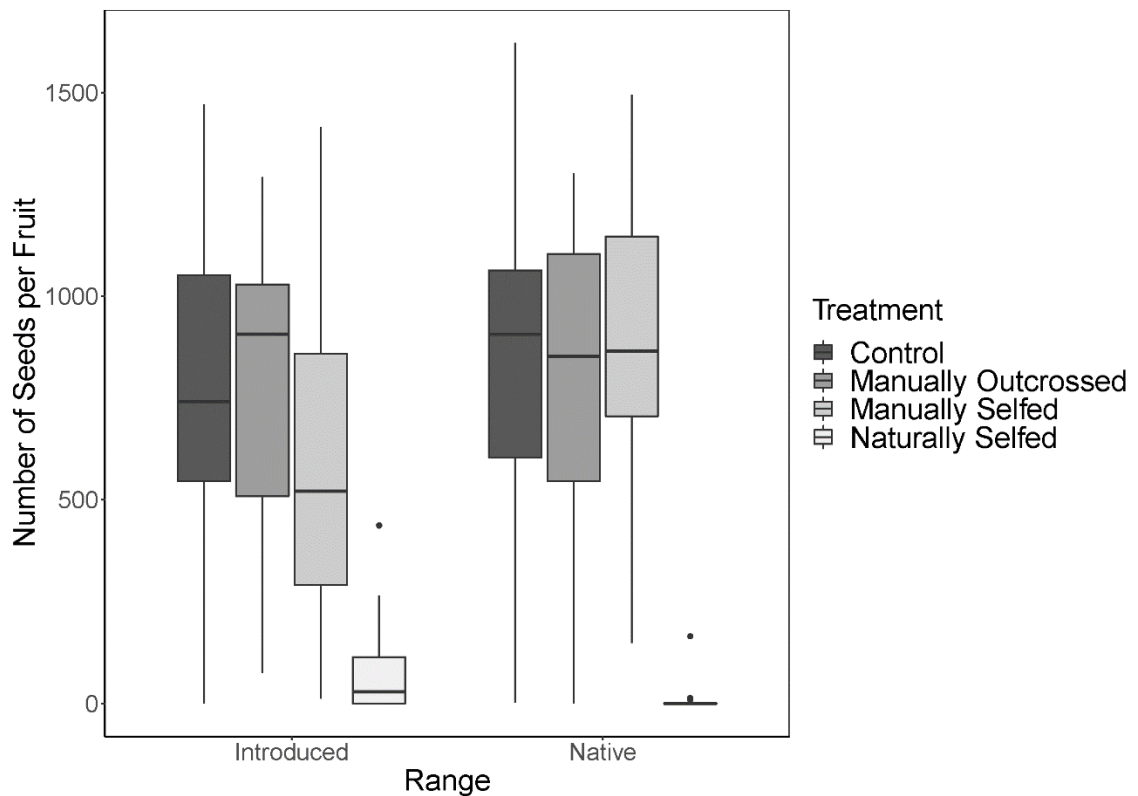
Figure 2.3. Average seed production after four hand pollination treatments applied to *Digitalis purpurea* flowers in the new range (Floresta, Colombia) and the native range (averaged for two populations in Britain: N = 8-20 plants per populations) See details of treatments in the text.

2.3.2 Pollinator assemblages and visitation

We quantified floral visitation from three-minute censuses that added up to 25-31 hours of observation per population in the non-native range (Floresta: 524 censuses, Choachí: 624, La Georgina: 506), and 7-10 hours in native populations (Calcot Wood: 140, Holy Cross: 201, Loder

Valley: 161). We ran more censuses in the non-native populations where visitor diversity was higher; plateaus in species accumulation curves show that with this sampling effort we were successful at recording an accurate representation of floral visitors in both native and tropical non-native populations (Appendix Fig. A2.4).

Populations in tropical mountains have a more diverse group of floral visitors with up to seven species of legitimate pollinators, compared to two in the native range (Appendix Fig. A2.4). Overall, bumblebees were the most frequent functional group of pollinators in all populations (Fig. 2.4), with a single *Bombus* species often dominating (Appendix Table A2.1). In non-native populations, hummingbirds performed up to 27% of the legitimate visits. Smaller bees and other insects were infrequent visitors in all populations; when they do visit, they often have trouble accessing the flowers due to the long hairs at the base of the corolla that act as barriers, or are too small to touch the stigmas and perform pollination.
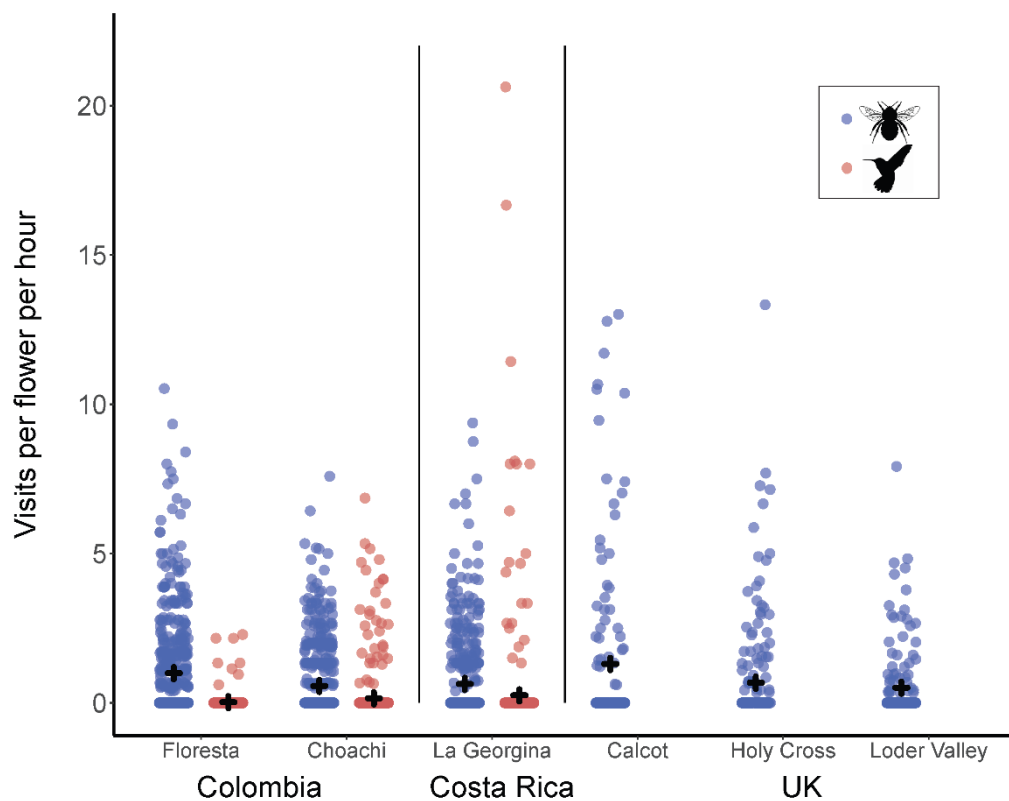
Figure 2.4. Visitation rates (number of visits/flower^{-1} x hour^{-1}) by bumblebees and hummingbirds on *Digitalis purpurea* in each study population. Circles represent different pollinator censuses and are plotted with a horizontal jitter to help visualising overlapping values. Black crosses show the mean values for each population; medians are very close to zero in all cases due to many censuses recording no visits

Populations in the introduced range received significantly more pollinator visits on average (0.86 ± 1.66 SD visits per flower per hour; N = 1654) than populations in the native range (0.79 ± 1.99 SD; N = 502; P = 0.005; Fig. 2.4). Flowers in the native range received significantly more visits by bumblebees on average than those in the introduced range (P = 0.003), with populations in Britain receiving a mean of 0.79 ± 1.99 SD visits per flower per hour and populations in the non-native range receiving 0.72 ± 1.38 SD bumblebee visits per flower per hour (Fig. 2.4). There was variation in visitation rates across populations within regions as well (Fig. 2.4).
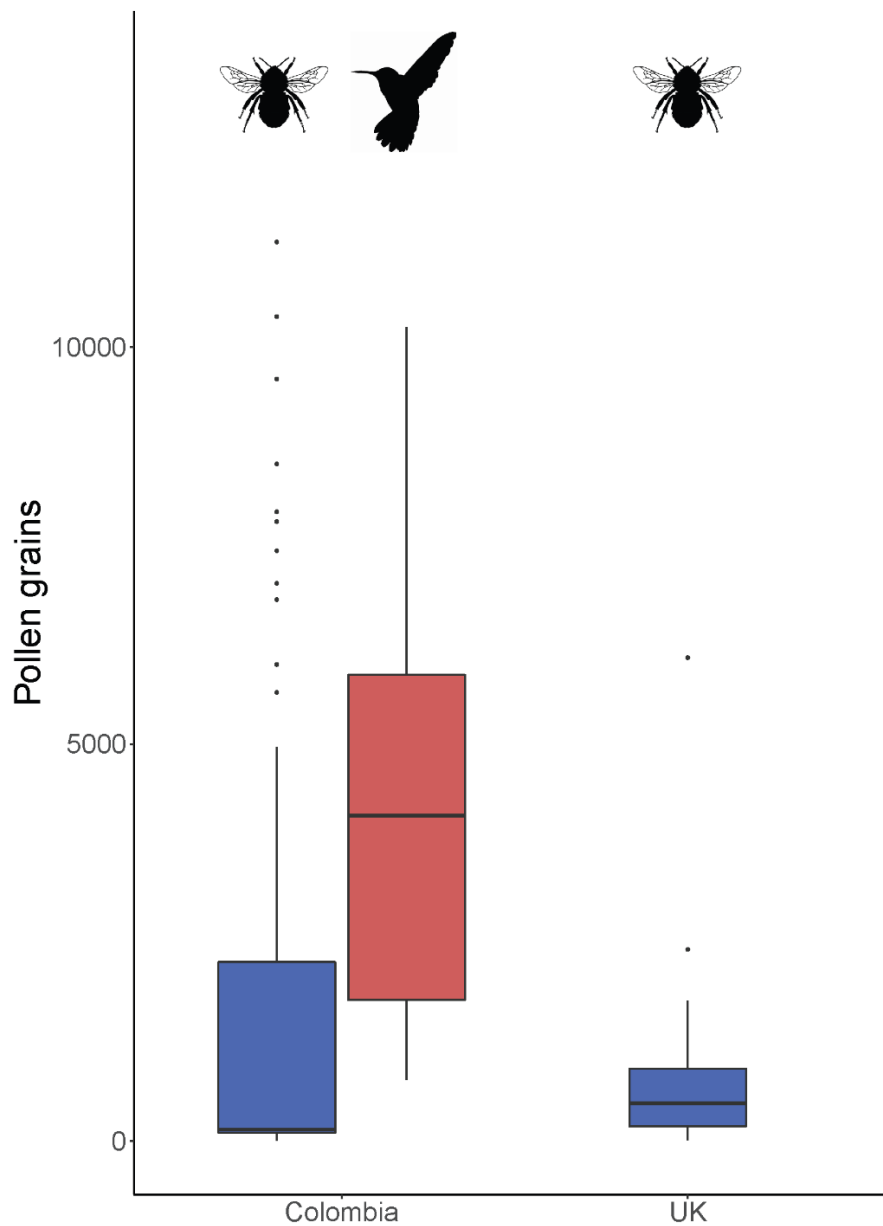
Figure 2.5. Pollen deposition on *Digitalis purpurea* stigmas after single visits by hummingbirds (red) in Colombia (N = 17) and bumblebees (blue) in Colombia (N = 95) and Britain (N = 38). Bumblebee icon modified from www.divulgare.net.

Measurements of functional morphological traits for *Bombus robustus* showed that they have an average tongue length of 6.9 mm ± 0.55 SD (N = 6). All other bumblebee species recorded in both ranges already have morphological measurements published in the literature (see summary in Appendix Table A2.2). The tongue length of bee species in the introduced range have means of 6.9 – 11.1 mm compared with 7.89 – 12.9 mm for those the native range (Appendix Table A2.2).

Nectar robbing by making holes at the base of the corolla was frequent in some of the non-native populations (for instance, 10.4% of all visits to flowers in Floresta), whereas it was absent in the native range (Appendix Table A2.1). Casual observations in Floresta found that 64% of plants had at least one flower robbed (N = 50), and 12% of plants in the sample had 100% of open flowers robbed (see Chapter 4). Some visitors acted both as pollinators and robbers. In some cases, pollinators switched from visiting flowers legitimately to robbing in the same foraging bout, and in others they performed a single foraging behaviour.

2.3.3 Effectiveness of pollinators

Hummingbird species of *Eriocnemis* and *Aglaeactis* in Choachí, Colombia, deposited a significantly larger number of pollen grains on average in single visits (4380 ± 2964 SD, N = 17) than bumblebees in native (728 ± 1053 SD, N = 38, adjusted P < 0.001) or non-native populations in Colombia (1780 ± 3179 SD, N = 95, adjusted P < 0.001; Fig. 2.5). When multiplying the number of pollen grains per visit by the visitation rate to find a coefficient of effectiveness, it shows that in Colombia bumblebees are deposit a higher number of grains per flower per hour (621) than hummingbirds (1354). Pollen deposition was variable within each functional group, but bumblebee data showed particularly large variation in the Colombian populations, from several hundreds to >13000 grains deposited in a single visit. This data comes from at least four different species of bumblebee (*Bombus funebris, B. hortulanus, B. rubicundus* and *B. robustus*) and could reflect local variation in flower abundance and also depend on the seasonal presence of large-bodied queens.

2.3.4 Comparisons of morphology and nectar traits

Whole corolla tube size did not differ significantly between the introduced (N = 783 flowers in 250 plants across seven populations) and the native range (N = 559 flowers in 165 plants in four

populations, estimate = 1.20; P = 0.51, Fig. 2.6a). By contrast, the proximal corolla tube was 13% and 26% larger on average (in Colombia and Costa Rica respectively) in the introduced range (N = 649 flowers in 201 plants and 146 flowers in 49 plants, respectively) as compared with the native range (N = 579 flowers in 166 plants; estimate = 0.84; P = 0.004; Fig. 2.6b). We found no significant differences between the native and introduced range for the volume of nectar (N = 31 and 142 plants respectively; estimate = 1.64, P = 0.22) or its concentration (estimate = 5.1, P = 0.31). Similarly, there were no significant differences between ranges in whole-plant vegetative traits: inflorescence height to the first flower (peduncle, N = 79 and 150 plants respectively; estimate = 7.92, P = 0.47) and rosette diameter (estimate = 7.46, P = 0.06; Appendix Fig. A2.5).

Overall, bigger plants (i.e. those with larger vegetative traits) did not consistently have larger corollas, across or within populations: whole corolla tube was significantly correlated with inflorescence height to the first flower in the complete dataset (r = 0.19, P < 0.01), but no other pair of vegetative and floral trait was significantly correlated. Interestingly, we found that plants in non-native populations produced, on average, 58.2% fewer flowers and fruits than plants in native populations (across population means: Britain = 124.1 fruits per plant with N = 76; Colombia = 53.0 flowers per plant with N = 95, Costa Rica = 48.8 with N = 34; P < 0.001) whilst producing the same mean number of seeds per fruit (P > 0.5). As a consequence, plants in the introduced range produce 64.2% fewer total seeds (P < 0.001). This is in spite of plants not being consistently smaller in the new range.

2.3.5 Natural selection on floral traits

We found no linear or quadratic selection on the whole corolla size in any population in the native or non-native range (Appendix Fig. A2.6). We found significant positive linear selection gradients on the size of the proximal corolla tube for all three non-native populations studied (Fig. 2.7, Table 2.2): in Floresta with a selection gradient of 0.32 (P < 0.001), in Choachí with a selection gradient of 0.14 (P = 0.008) and in La Georgina with a selection gradient of 0.22 (P = 0.04). In contrast, there was no evidence of selection on proximal corolla tube size in Britain populations (Fig. 2.7; Table 2.2; Appendix Fig. A2.7), indicating that the proximal corolla tube is not under selection in the native region. Non-linear analysis showed no evidence for stabilising or disruptive selection on the proximal corolla size in any population (Table 2.2).
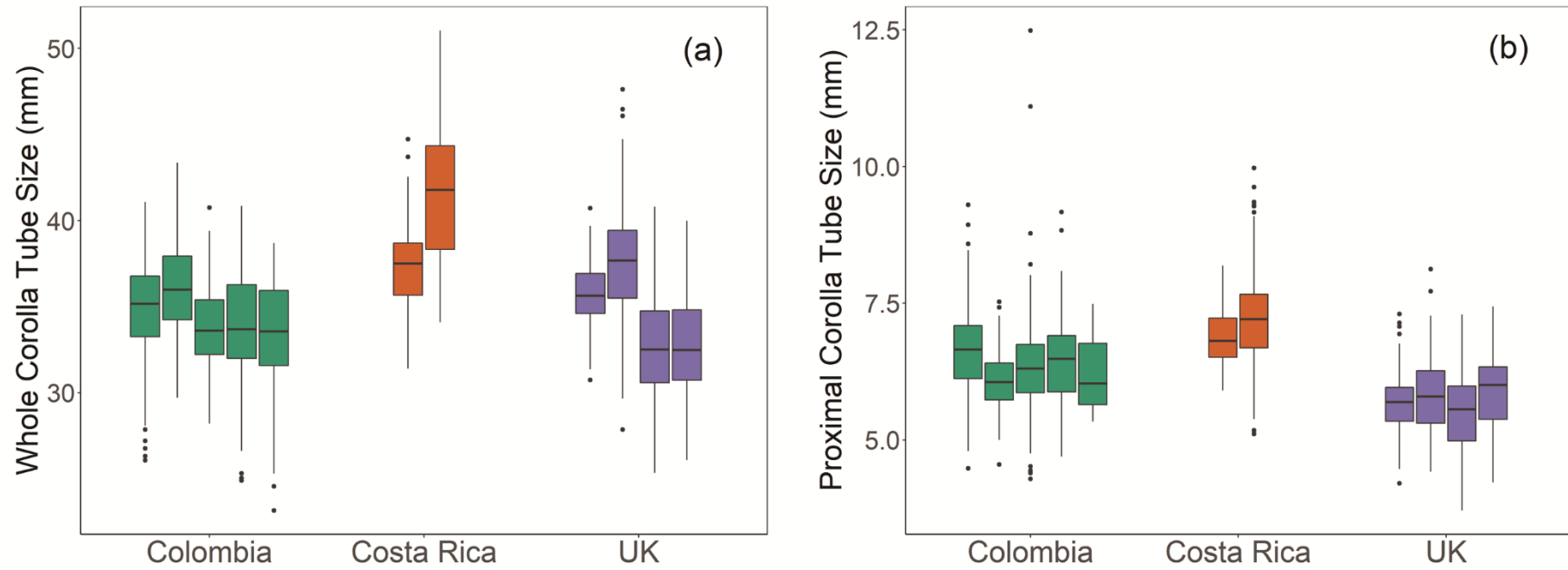
Figure 2.6. Comparison of (a) whole corolla tube size and (b) proximal corolla tube for all *Digitalis purpurea* populations (each in a separate boxplot) in the three regions
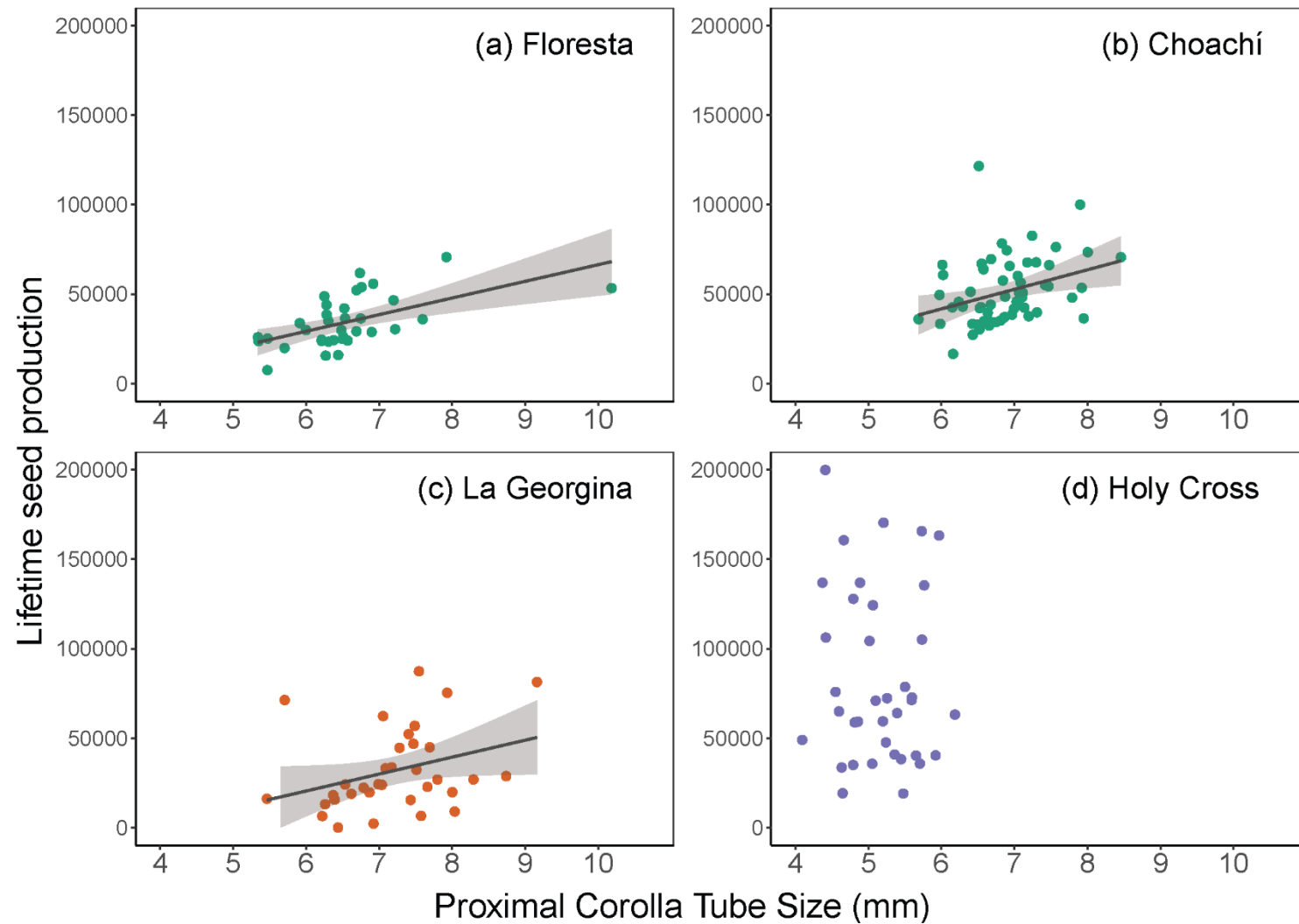
Figure 2.7. Lifetime seed production (numbers of seeds) against proximal corolla tube size, measured as the geometric mean of proximal corolla tube length and width. Non-native populations of *Digitalis purpurea* (a) Floresta, Colombia, (b) Choachí, Colombia, (c) La Georgina, Costa Rica; and native (d) Holy Cross in Britain.

The selection analysis in the previous paragraph used the geometric means of corolla traits, as explained in the Methods. To get further insight on which aspect of the corolla is the target of selection, we ran separate univariate selection models for the height and the length of the whole corolla and the length and width of the proximal corolla tube in each population. We found that selection is concentrated mostly on the length of the proximal corolla; it was significant in all three non-native populations (Table 2.2; Floresta: selection coefficient 0.27, P < 0.001; Choachí: selection coefficient of 0.10, P = 0.03; La Georgina: selection coefficient 0.21, P = 0.04), and significant on the width of the proximal corolla in only one of them (Floresta: selection coefficient 0.14, P = 0.02). For the whole corolla, univariate models found significant selection only on the height in one non-native population (Choachí: selection coefficient of -0.10, P = 0.03; Appendix Table A2.3).

We found variable evidence of selection on nectar traits in the non-native populations (Table 2.2). Nectar volume was under strong positive linear selection in one Colombian population (Choachí: selection gradient 0.16, P < 0.001), but not in the other populations. Nectar concentration was not under linear or non-linear selection in any non-native population either. Native populations were not included in this analysis, as we did not measure nectar traits in the same individual plants where we estimated seed production.

2.4 Discussion

We found evidence of evolution in floral morphology when comparing *Digitalis purpurea* populations in the native range with two recently colonised regions. Plants in the new range, visited by a pollinator assemblage that includes a new functional group – hummingbirds – and novel bumblebee species, had proximal corolla tubes that are 13 to 26% larger than plants in the native range, where they are pollinated solely by bumblebees. This corolla trait plays a role in determining access to nectar, as only pollinators with long enough tongues or beaks can reach the nectaries at the base of the ovaries. Consistent with this morphological change, we also found directional selection acting to increase the proximal corolla length in the three populations we tested in the new range, whereas corolla traits in the native range are not currently under detectable selection. Our study is the first to investigate contemporary evolution of flowers after the addition of a new functional pollinator group, and we find changes that are consistent with rapid evolutionary change in response to the new pollinator environment.

Table 2.2. Directional and quadratic selection gradients or coefficients (±SE) for the floral traits in each population of *Digitalis purpurea*. Values that are statistically significant from zero are in bold type with p values indicated by *(p < 0.05), **(p < 0.01) and ***(p < 0.001)

Trait	Country	Population	Directional (β)	Non-Linear (γ)
Whole Corolla Tube Size	Britain	Loder Valley	-0.21 ± 0.124	0.00 ± 0.000
	Britain	Holy Cross	0.00 ± 0.111	0.00 ± 0.000
	Costa Rica	La Georgina	0.05 ± 0.125	0.00 ± 0.000
	Colombia	Floresta	-0.03 ± 0.064	0.00 ± 0.000
	Colombia	Choachí	-0.07 ± 0.049	-0.06 ± 0.046
Proximal Corolla Tube Size	Britain	Loder Valley	0.21 ± 0.119	0.00 ± 0.001
	Britain	Holy Cross	-0.05 ± 0.106	0.00 ± 0.000
	Costa Rica	La Georgina	**0.22 ± 0.131***	0.00 ± 0.000
	Colombia	Floresta	**0.32 ± 0.080***	-0.02 ± 0.046
	Colombia	Choachí	**0.14 ± 0.043**	0.00 ± 0.000
Proximal Corolla Tube Length	Britain	Loder Valley	0.15 ± 0.123	0.00 ± 0.000
	Britain	Holy Cross	-0.02 ± 0.096	0.00 ± 0.000
	Costa Rica	La Georgina	**0.21 ± 0.13***	0.00 ± 0.000
	Colombia	Floresta	**0.27 ± 0.058***	-0.01 ± 0.008
	Colombia	Choachí	**0.10 ± 0.048***	0.00 ± 0.000
Proximal Corolla Tube Width	Britain	Loder Valley	-0.03 ± 0.118	0.00 ± 0.000
	Britain	Holy Cross	-0.07 ± 0.102	0.00 ± 0.000
	Costa Rica	La Georgina	0.20 ± 0.127	0.00 ± 0.000
	Colombia	Floresta	**0.14 ± 0.067***	0.00 ± 0.000
	Colombia	Choachí	0.06 ± 0.045	-0.03 ± 0.022
Nectar Volume	Costa Rica	La Georgina	-0.10 ± 0.128	0.00 ± 0.000
	Colombia	Floresta	-0.05 ± 0.075	0.00 ± 0.000
	Colombia	Choachí	**0.16 ± 0.046***	0.01 ± 0.012
Nectar Concentration	Costa Rica	La Georgina	0.06 ± 0.135	-0.09 ± 0.072
	Colombia	Floresta	0.06 ± 0.070	0.00 ± 0.000
	Colombia	Choachí	0.05 ± 0.044	0.01 ± 0.006

Evidence of population differences in corolla morphology consistent with variation in pollinator morphology is abundant in the literature; examples include, i) *Gladiolus longicollis* corolla tube ecotypes that are maintained by differences in the local moth community (Anderson et al., 2010), ii) *Nicotiana glauca* populations with dissimilar corolla lengths when visited by hummingbirds with correspondingly different bill lengths (Nattero et al., 2011), and iii) *Narcissus papyraceus* populations with larger tepal and corolla tubes when visited by moths rather than syrphid pollinators (Pérez-Barrales et al., 2007). Our *D. purpurea* data show that these adaptive changes can take place rapidly, in the short time since colonisation in the introduced range (< 85 generations). In this case, larger proximal corolla tubes might be favoured by an improved efficiency of pollen transfer by novel pollinators. The bumblebee species in the new range have similar functional morphological traits to the bees in the native range, as their tongue lengths and body sizes do not differ significantly (del Castillo & Fairbairn, 2012; see also Appendix Table A2.2). However, hummingbirds constitute an important functional group in the new range, where they perform on average 22% of the visits and are more effective than bumblebees at depositing pollen on stigmas. Hummingbirds have been shown in the past to be more effective at delivering pollen to other flowers compared to bumblebees, even if they remove the same amount, making them overall more effective pollinators (Castellanos et al., 2003). The birds could thus be exerting selective pressures for easier access to *D. purpurea* nectar and better morphological fit while hovering, i.e. plants that have longer and less constricted proximal corolla tubes. Here we do not yet provide direct evidence of this mechanism for selection by the hummingbirds, but will be testing it with selective exclusion of pollinators in the future. Interestingly, we found no differences or selection acting on the whole corolla tube, suggesting that fit and access to nectar rewards is determined mainly by the proximal base of the corolla in this species. Selection on this proximal part of the corolla is consistent across distant populations, even though there is no pollen limitation for seed quantity in any of them. One possible reason for selection to be occurring in the absence of pollen limitation is that hummingbirds could also be enhancing seed quality by reducing geitonogamy (when a flower is fertilised by pollen from a flower on the same plant) if pollen is moved farther from the parental plants, as seen in multiple bird-pollinated plants (reviewed by Krauss et al., 2017; Pauw, 2019). This aspect and a potential effect on the male components of reproductive success are yet to be tested in this species.

These findings are consistent with patterns of selection typically imposed by hummingbirds and other bird pollinators, who have favoured the evolution of flowers with long corollas across multiple angiosperm lineages, in one of the best examples of floral evolutionary convergence

(Grant & Grant, 1968; Fenster et al., 2004). We found no differences between native and introduced populations in other floral traits that are often associated to hummingbird pollination, such as nectar volume or quality. Overall, *D. purpurea* flowers produce large enough volumes of nectar to be attractive to hummingbirds (3.8–7.4 µl in 24h without visitation, pers. *obs*.). There was significant directional selection for higher nectar volume in one non-native population, but this was not consistent in all studied populations. Because the hummingbird communities visiting *D. purpurea* are different in each population, this warrants further investigation in the future. However, nectar traits are highly sensitive to environmental conditions (e.g. water availability, temperature, etc; reviewed in Parachnowitsch et al., (2019)) and are thus likely to require long-term consistent selection for a detectable response. This is in contrast to linear morphological traits that often present high values of heritability, even when measured in field conditions (Ashman & Majetic, 2006; Castellanos et al., 2019), and that have been shown to change in response to single mutations with implications for pollinator visitation (see Ding et al., (2017) for an example in *Mimulus*). For *D. purpurea* we are in the process of measuring heritability both in the field (using molecular markers) and in a common garden, and preliminary results from the field studies point towards very high and significant narrow sense heritabilities ($h^2 > 0.45$) for linear corolla traits (Castellanos et al., unpublished; also see Chapter 3). This is consistent with the rapid evolution observed in the newly colonised populations.

The capacity for rapid evolution in corolla traits has been corroborated by several studies which imposed artificial selection under greenhouse conditions and found a quick response, including changes to corolla diameter, area (Worley & Barrett, 2000; Lendvai & Levin, 2003; Lehtilä & Holmén Bränn, 2007) and length (Conner et al., 2011). Evidence of very rapid evolution in natural conditions, (faster than ecotype formation in species with large home ranges), however, has been mostly limited to other reproductive traits such as flowering phenology (Colautti & Barrett, 2013; Lustenhouwer et al., 2018), as well as self-compatibility to provide reproductive assurance for plants losing pollinators when invading a new area (Barrett et al., 2008; note that in self-compatible *D. purpurea* there is no change in the breeding system in the newly colonised area, where dependence on pollinators remains high). Examples of rapid evolution of corolla traits in the wild are scarce, with a remarkable exception provided by Campbell et al., (2018). Studying an *Ipomopsis* hybrid zone, they demonstrated contemporary evolution of corolla length in approximately five generations. Notably, these changes closely followed their predictions of evolutionary change based on previous measures of the strength of selection by pollinators and the high heritability of corolla length. Flowers in this species are under divergent selection along

an altitudinal cline, where pollinator communities vary along the cline and impose varying selection regimes.

Although studies showing rapid evolutionary change in corolla traits in the wild are rare, several others have demonstrated short-term changes in selection after disruption of previous pollination environments. Temeles and Bishop (2019) measured natural selection on corolla length in *Heliconia wagneriana* in Dominica before and after Hurricane Maria, which changed the composition of the plant community and as an indirect consequence, the prevalent hummingbird visitor of *H. wagneriana* from one year to the next. Before the hurricane, plants experienced no selection on corollas, but after a short-billed hummingbird became the predominant pollinator, the morphological mismatch led to strong selection for shorter corollas. Similarly, a study by Murúa et al., (2010) showed how the predominant pollinators of a wild violet differ between human-transformed forests and native forests less than 4 km away. The change in pollinators has led to relaxed selection on flower number and novel disruptive selection on corolla shape in the disturbed sites. If sustained over a long time period, the change in selective pressures documented in these two study cases could potentially lead to floral evolution at a local scale.

In both previous examples it is certain that the evolving populations are the same or part of the original ones. However, in cases of long-distance colonisation there is potential for founder effects arising from the invasion process if, for example, only a subsample of the original phenotypes were introduced to the new range. Changes in corolla length of invasive *Nicotiana glauca* populations studied by Schueller (2007), for instance, could not be explained by changes in the pollinators alone and were instead consistent with an initial bottleneck. In the case of our *D. purpurea* study populations, three sources of evidence suggest that the observed changes in the proximal corolla tube are not simply the consequence of a stochastic founder event. First, the comparison of a set of ~9K SNP markers shows that populations in Colombia and Costa Rica are genetically distinct from each other and each closer to those in Britain (see Methods; Castellanos, in prep.). This is consistent with separate introductions to the two tropical regions and the convergent change in proximal corollas. Second, we found no significant differences in vegetative traits between the native and introduced range. If present, differences in a number of traits could have suggested founder effects with phenotypically distinct plants colonising the new areas, or divergent evolution after invasion, but none seem to be the case in these tropical regions. Willis et al., (2000) also studied vegetative traits in non-native populations of *D. purpurea* in Australia and New Zealand, and found that growth traits showed no differentiation from British and French populations after a post-invasion period similar to the one in this study.

Vegetative traits in *D. purpurea* thus appear to vary little even when plants successfully colonize different continents. Finally, no other floral trait that we studied (whole corolla tube and nectar traits) showed differentiation between native and non-native populations, nor experienced significant selection across the non-native populations. A parallel change in whole corolla tube could be expected, since it is correlated to the proximal part of the corolla to some extent and corolla traits tend to be highly integrated (Berg, 1960). Strong selection could be decoupling the evolution of the two parts of the corolla; however, testing this hypothesis will require further experimental work in the non-native populations.

2.4.1 Concluding remarks

Our study adds to many previous studies that use range changes as an opportunity to study trait evolution in plants. Our findings also contribute to the growing evidence that plants invading new areas can rapidly evolve even after only decades since their establishment (Colautti & Lau, 2015), potentially favoured by genetic isolation from the original populations, and in spite of potential constraints such as genetic correlations among traits (Ashman & Majetic, 2006). Here we demonstrate that range changes can also be used to study reproductive resilience and floral evolution when new pollinators are acquired. Additions of new functional groups to a plant´s pollinator environment are likely to happen more often as plants or pollinators migrate due to human influence, but it is also presumably a common feature in the long-term evolution of the angiosperms (Grant & Grant, 1965; Stebbins, 1970). During episodes of contact with new pollinators, even in the presence of previous ones, novel and creative selection can change the tempo of flower evolution (reviewed by Harder & Johnson, 2009). By focusing on a period of potential floral innovation, our study on *D. purpurea* shows that adaptation of key floral traits to new pollinators can happen rapidly in response to sustained selection. Further studies on contemporary evolution in plants acquiring novel pollinators can add more evidence to confirm that selection for novel phenotypes followed by rapid evolutionary change can be an important force behind the extraordinary diversity of flower form and function.

Chapter 3

Heritability of floral traits in native and introduced populations of *Digitalis purpurea*

Abstract

Predicting the evolutionary response to selection can be a useful tool for studying the evolutionary trajectory of plants and all organisms, and methods to estimate the change in a trait require calculation of the heritability of the trait. In this chapter we use *Digitalis purpurea* as a model system, a plant with demonstrable rapid evolution of floral traits in introduced populations, to assess the sources of variation controlling the traits and to investigate whether plants are locally adapted to their populations of origin. For this chapter we collected seed from native and introduced populations and grew plants in a common garden experiment. Following flowering, we measured floral traits including corolla weight, whole corolla size, proximal corolla length and proximal corolla width. We tested for evidence of local adaptation by comparing floral trait expression for populations between the common garden and field conditions, and used variance partitioning to assess how the variation in each population was distributed. We also used parent-offspring regression for plants in the common garden, and found high heritabilities for floral traits in populations, which is in line with the ability to rapidly evolve. Floral trait values tended to cluster closer together around a common mean in the common garden as compared with the natural conditions of field populations, indicating an environmental component to the trait variation. In addition, trait means tended to be higher in the garden for plants sourced from Britain, whereas plants sourced from introduced populations tended to have lower means in the common garden than in the field. We argue that this indicates a local adaptation of plants to environmental conditions of their populations of origin.

3.1 Introduction

It is expected that the variance underlying morphological floral traits has a strong genetic influence (Ashman & Majetic, 2006; Geber & Griffen, 2003). Our expectation of at least some level of genetic control to floral traits (Campbell, 1996; Opedal, 2019) is in part due to their involvement in ensuring reproduction success, such as through pollination attraction and interaction. Floral trait groups with a high heritability include floral volatiles (Zu et al., 2015), phenological traits (Elzinga et al., 2007), herkogamy (spatial separation of reproductive parts), floral display traits and morphological corolla traits (Opedal, 2019). Floral morphological traits in particular are expected to have a large proportion of the underlying variation controlled by additive genetic variance, since reproductive assurance typically relies on specific and accurate pollinator attraction and interaction to transfer pollen efficiently (Armbruster et al., 2009; Ne'eman et al., 2010). Corolla tube length, for instance, has high but wide-ranging narrow sense heritabilities of between 0.17 to 0.77 (Table S3 in Opedal, 2019; N = 10 studies) and the evolvability also tends to be high. Evolvability is the expected evolutionary response to selection standardised by the mean (Hansen et al., 2011), whilst heritability is the proportion of the variation underlying the trait due to additive genetic variance (Lynch & Walsh, 1998).

In this study we focus on the estimation of heritability, measured from the similarity of individuals with a known relatedness. Heritability provides a useful metric when combined with the strength of selection (such as in the breeder's equation, $R = S * h^2$, where R is the response to selection, S is the strength of selection and h^2 the heritability) since this can predict the response to selection and resulting evolutionary change (Grant & Grant, 1995; Morrissey et al., 2010; Walsh & Lynch, 2014). However, there have not been many studies combining estimates of heritability with the strength of selection. In addition to this, no study has of yet estimated the heritability of a floral trait that has also been shown to have demonstrable rapid evolution in wild populations. A significant and high heritability of floral morphological traits would be consistent with the potential to rapidly evolve in response to new selective pressures. Here, we focus on a plant with observed rapid selection on and resulting rapid evolution of a floral trait following novel pollinator acquisition (Mackin et al., 2021; see Chapter 2), and we estimate the heritability of corolla traits in this study system to find whether traits showing rapid evolution are indeed highly heritable.

Recent work on *Digitalis purpurea*, the common foxglove, found contemporary and rapid evolution of a part of the corolla tube (Chapter 2). This work on introduced populations of *D.*

purpurea in tropical mountains in the Americas (in Colombia and Costa Rica) found that following range expansion the plant had rapidly evolved larger proximal corolla tubes alongside pollination by an additional new functional pollinator group, hummingbirds. Consistent with differences in this trait between parts of the range, the plants were under directional selection for larger proximal corolla tubes in the introduced populations only (Mackin et al., 2021). Previous studies of corolla trait heritability in other species suggest that in this case, the rapid response to evolution we observe is not only explained by the strong selection occurring in the new environment, but also aided by high pre-existing levels of heritable variation. Here, we reared *D. purpurea* in a common garden experiment to quantify the proportion of the variation underlying the floral traits that is controlled by additive genetic variance and to what extend the traits are influenced by environmental variation.

Common garden experiments can also be a useful tool for studying local adaptation in plants sourced from different populations. In the focal plant described above, after a number of generations we would expect further adaptive differentiation in the geographically isolated populations, through divergent natural selection and genetic drift (Kawecki & Ebert, 2004). The two ranges of *D. purpurea* studied in previous research (Britain and Central/South America) differ considerably in biotic and abiotic conditions. Beyond pollinator differences, we could expect differences in climactic conditions (temperature, rainfall etc), seasonality, herbivory and disease to have shaped the evolutionary trajectory of the floral traits since reproductive isolation. For instance, if plants living in warmer climates invest more in defence due to higher herbivore abundance, then reproductive output may be comparatively lower when grown at different temperatures (Kawecki & Ebert, 2004). Part of this study will test for evidence of local adaptation by comparing the floral trait expression of plants sourced from different ranges in the southern England-based common garden.

In this study we examined the trait variation expressed when plants originating from different populations were grown in a standardised environment. If traits are under strong genetic control, then we expect that differences in traits should persist when plants are grown in a common garden environment (Bossdorf et al., 2005; Colautti et al., 2009; Williams et al., 2008). To quantify the heritability of floral morphological traits we ran parent-offspring regressions in common garden plants across populations. We expect that the genetic components of variation for morphological traits could be large, in line with typical findings for floral morphological traits. In addition to this, we compared the floral trait expression of plants sourced from introduced populations and native populations in the southern England-based common garden as an indicator of local adaptation.

3.2. Materials and Methods

3.2.1 Study System

Digitalis purpuea, the common foxglove, is a facultatively biennial herb native to European woodlands, with non-native naturalised populations in tropical mountains in Central and South America (among other locations around the world). The plant commonly grows in dense aggregations, often following disturbance to the seed bank, and they produce showy inflorescences of many dozen purple bell-shaped flowers. In tropical environments, different populations in the same area can flower at different times of the year, but in temperate forests the plant typically flowers between May and July of the second summer of growth. The flowers are protandrous, and anthers dehisce shortly after anthesis whilst the stigmas become receptive up to five days after. Although the plant is self-compatible and is capable of producing small amounts of seeds by self-pollination (Grindeland, 2008), animal pollination is required for full seed set and is the predominant breeding system strategy (Mackin et al., 2021). The plant secretes copious amount of nectar deep inside bell shaped flowers, which is utilised as a resource by pollinating animals in most parts of the range.

Populations in the plant's native range of Europe are pollinated solely by bumblebees, with *Bombus hortorum* and *B. pascuorum* in Britain being predominant visitors (Grindeland et al., 2005; Broadbent & Bourke, 2012). Recent research (see Chapter 2) found that introduced populations in Central and South America were pollinated by hummingbirds and bumblebees. This work found a correlation between the inclusion of hummingbird visitors in the pollinator assemblage and an increased size of the proximal corolla tube (the part that restricts access to the nectaries). This research demonstrated rapid evolution of this trait during the <85 generations in the introduced areas (Mackin et al., 2021). We also measured the size of the whole corolla but found no selection on this trait or differences between ranges.

3.2.2 Common garden Setup

We grew plants from non-native and native populations, using seed sourced from two populations in Britain (Holy Cross and Loder Valley) in 2016, from Floresta in Colombia in 2017 and from La Georgina in Costa Rica from 2018 (Table 3.1). From approximately 40 individuals in each population (referred to as "mothers" hereon), ripe fruits were collected from inflorescences and stored in glassine envelopes. We measured floral morphology for each

mother using the methodology described below for offspring. Fruits were stored in a cool, dry and dark room until they were needed for sowing.

We grew six offspring (half-sibs) from 15 mothers (hereon referred to as "family") per population, giving us 6 x 15 x 4 (= 360 plants in total) in total growing at once. Seed was sown in 15cm diameter (1.5L) pots, with families from each of the populations assigned to positions on a gravel tray randomly. For the seedling stage, positions of trays in the greenhouse were randomised inside blocks and sub-blocks. Initial positioning of trays and position of pots within trays were determined by a random number generator. Care was taken when handling seeds during sowing to reduce the likelihood of contamination.

Once plants had grown to an appropriate size they were transplanted to 21cm diameter (4L) pots in the summer of 2018. During November 2018 *D. purpurea* plants were kept outside for vernalization (cold treatment) until March 2019 and flowered in the next Spring in 2020. Between May and July we collected flowers from 198 flowering plants across all population sources. Fewer plants from the La Georgina population grew to the flowering stage, resulting in La Georgina being underrepresented in the heritability analysis.

3.2.3 Trait Measurements

We measured the four floral traits included in Mackin et al., (2021; see also Chapter 2) that include the length and height of the whole corolla (when pressed), and the length and width of the "proximal corolla tube" (the part that restricts access to the nectaries), and also the dry weight of complete corollas. Between two and six flowers were collected from each individual in the study, so intra-plant variation in floral traits (e.g. such as different heights of the inflorescence) could be considered and included in the analysis. Picked flowers were collected and immediately pressed in filter paper and a metal press. Flowers were then dried in an oven at 45ºC for at least 48 hours, and for further 24 hours immediately before weighing. Whole corollas were weighed in a precision balance to the nearest 0.001g, mounted on paper and scanned at a high resolution. Weights of the corolla reflect the biomass and overall size of the flowers, whilst the linear measures represent the two-dimensional size of a pressed flower.

We then used digital images of pressed flowers to measure whole corolla length, whole corolla height, proximal corolla tube length and proximal corolla tube height, using ImageJ software. We tested whether these linear traits are significantly correlated with each other, using the "chart.Correlation" function from the package *PerformanceAnalytics* in R (Peterson et al., 2018).

Similar to the field populations, we found that whole corolla length and whole corolla width covaried significantly in the common garden (r = 0.49 across all populations, up to r = 0.62 in La Georgina; P < 0.001) We thus combined these measures into a geometric mean for analysis ("whole corolla size") (as in Mackin et al., 2021). Corolla weight also correlated with whole corolla size with a strong correlation coefficient (r = 0.64 across all populations; P < 0.001) but was included by itself in the analysis as a comparison for how the heritability of the biomass differs from the former linear measurement of size. Proximal corolla length and proximal corolla width had a significant but weak correlation in the common garden (r = 0.12 overall; P < 0.01; up to r = 0.21 in Floresta) so these traits were analysed separately in this study (in Chapter 2 these traits were analysed as a combined trait and also separately). We only included flowers that had been pressed properly without any breaks or improper folding in the analysis.

Table 3.1. Populations that *Digitalis purpurea* plants were sourced from, for use in this study.

Region	Population	Coordinates (latitude, longitude)	Elevation (m)
Britain	Loder Valley	51.055, -0.093	98
	Holy Cross	50.972, 0.199	128
Colombia	Floresta Reserve	4.802, -73.998	3050
Costa Rica	La Georgina	9.559, -83.724	3070

3.2.4 Trait variation in the common garden experiment

To examine the floral trait variation within each population we use variance partitioning to find the variance components at each level of analysis. We used 'lme' (from the *nlme* package) to find the proportion of variance (i) among families within populations; (ii) among plants within families and (iii) among flowers within plants for each population. We repeated this procedure for each floral trait, for each population of origin.

3.2.5 Comparing common garden and field populations

To find evidence of local adaptation, we compared the floral trait variation for populations grown in the common garden with the field populations from which they were sourced. For instance, if plants from native populations show higher trait values in the common garden but introduced populations do not, then this could be evidence for local adaptation. We used mixed-effects models with "plant individual" nested within "family" as random factors to compare floral trait means in the field populations with their corresponding group in the common conditions of the garden.

We tested whether the floral trait variation clusters more closely around a common mean across all four populations when plants are reared in the standardised conditions of the common garden setting rather than in the field. For this we first used a Shapiro-Wilk test to confirm that for most floral traits data were non-normally distributed ($P < 0.001$), apart from proximal length in the common garden ($P = 0.176$). Thus, we used a Bartlett's test (from the *stats* package) for proximal length and a Levene's test (using the *car* package) for all other traits to check for equal variances across all field plants and all common garden plants. If some of the variance in corolla traits is a response to environmental conditions, then we would expect convergence of floral trait values around a mean.

3.2.6 Heritability estimates

We used parent-offspring regression to estimate the narrow sense heritability of floral traits. We ran linear regressions for each floral trait in common garden plants, to regress offspring values against values of the parent. Floral trait values were averaged within each individual and compared with parents across all populations for whole corolla size, corolla weight, proximal corolla length and proximal corolla width. We used mixed-effects linear models ('lmer') from the packages *lme4* and *lmerTest*, with population as a random factor in R. We estimated heritability as the slope of the regression line, multiplied by two because we only have values for one parent (Fernandez & Miller, 1985; Smith & Kinman, 1965). We combined data for all populations for the analysis as the offspring are reared within the same common environment. We did not include family as an additional random factor in addition to the population, as this did not significantly improve the fit of the model when comparing AIC values.

Table 3.2. Floral trait variation for each population in the common garden. Values included are the means, standard deviation (SD) and sample size (N) of flowers (and plants in brackets) in the field and plants in the common garden (garden) and the P-value from a linear model comparing the two groups. Colours of P value indicate whether mean values increased (Green) or decreased (red) between the field and the common garden groups.

Population	Value	Corolla Size (mm)	Corolla Weight (mg)	Proximal Length (mm)	Proximal Width (mm)
Loder Valley	Field Mean, SD	32.74 ± 2.73	31.51 ± 6.55	5.33 ± 0.81	6.58 ± 0.89
	Field N	112 (40)	120 (40)	120 (40)	120 (40)
	Garden Mean, SD	37.13 ± 4.09	36.05 ± 7.17	5.88 ± 0.99	6.87 ± 0.79
	Garden N	174 (62)	189 (62)	180 (62)	180 (62)
	P value	**<0.001*****	**0.001*****	**<0.001*****	**0.026***
Holy Cross	Field Mean	31.76 ± 2.36	32.08 ± 5.88	4.91 ± 0.83	5.41 ± 0.67
	Field N	112 (39)	120 (40)	119 (40)	119 (40)
	Garden Mean	36.92 ± 2.97	34.02 ± 6.61	5.77 ± 0.95	6.50 ± 0.74
	Garden N	165 (54)	120 (55)	164 (55)	164 (55)
	P value	**<0.001*****	0.095	**<0.001*****	**<0.001*****
Floresta	Field Mean	33.77 ± 2.55	38.21 ± 7.25	5.46 ± 1.07	7.77 ± 1.12
	Field N	117 (40)	120 (40)	120 (40)	120 (40)
	Garden Mean	33.64 ± 2.60	29.48 ± 5.51	4.57 ± 0.89	6.62 ± 0.68
	Garden N	125 (48)	176 (57)	133 (49)	133 (49)
	P value	0.748	**<0.001*****	**<0.001*****	**<0.001*****
La Georgina	Field Mean	41.48 ± 3.87	45.37 ± 9.81	7.31 ± 1.23	7.19 ± 0.95
	Field N	116 (39)	117 (39)	116 (39)	116 (39)
	Garden Mean	40.26 ± 3.48	40.81 ± 6.53	6.62 ± 1.12	6.49 ± 0.67
	Garden N	67 (22)	74 (23)	71 (23)	71 (23)
	P value	0.149	**0.017***	**0.012***	**<0.001*****

3.3. Results

3.3.1 Trait variation within the common garden experiment

We find mixed results for the variance partitioning, with different parts of the range showing different patterns. For plants sourced from populations in the native range (Loder Valley and Holy Cross) the variation in corolla size, corolla weight and proximal corolla length and proximal corolla width was split between all levels of analysis; across families, among plants within a family, and among flowers within plants (Fig. 3.1). However for introduced populations (Floresta and La Georgina), a high degree of variation was both among and within plants, and less across families in a population (Appendix Table A3.1).

3.3.2 Comparing common garden and field populations

We compared trait values in field populations with the parental population as an indicator of local adaptation. For populations in Britain, floral traits tended to be larger in the common garden than in the field population. For Loder Valley plants, floral traits were significantly larger in the common garden than in the parental field population, including whole corolla size ($P < 0.001$), corolla weight ($P = 0.001$), proximal corolla length ($P < 0.001$) and proximal corolla width ($P = 0.026$; Table 3.2; Fig. 3.2). Similarly, in Holy Cross we found that whole corolla size ($P < 0.001$), proximal corolla length ($P < 0.001$) and proximal corolla width ($P < 0.001$) were significantly larger in the common garden than in the field population plants, but corolla weight was not different ($P = 0.095$; Table 3.2; Fig. 3.2). The introduced populations showed the opposite trend. In Floresta and La Georgina, corolla weight, proximal corolla length and proximal corolla width were found to be larger in the field populations than in the common garden, but not for whole corolla size (Table 3.2).

Tests for equal variances showed that variation in all floral traits clusters more closely together in the common garden plants than in field population plants. We found that whole corolla size had more variance in the field plants (mean = 34.98mm, $\sigma^2 = 23.52$, N = 457; $P < 0.001$) than in the common garden (mean = 36.64mm, $\sigma^2 = 15.15$, N = 531). Similarly, plants showed more variance in corolla weight in the field populations (mean = 36.74, $\sigma^2 = 87.17$, N = 477; $P < 0.001$) than in the common garden plants (mean = 34.16, $\sigma^2 = 54.67$, N = 611). Values for the proximal corolla tube length cluster more closely together in the common garden (mean = 5.63, $\sigma^2 = 1.37$, N = 548; $P = 0.001$) than values for the field data (mean = 5.74, $\sigma^2 = 1.83$, N = 475). Similarly, values for the proximal corolla tube width cluster more closely together around a mean in the

common garden (mean = 6.65, σ^2 = 0.56, N = 548; P < 0.001) than for the field population plants (mean = 6.74, σ^2 = 1.61, N = 475).
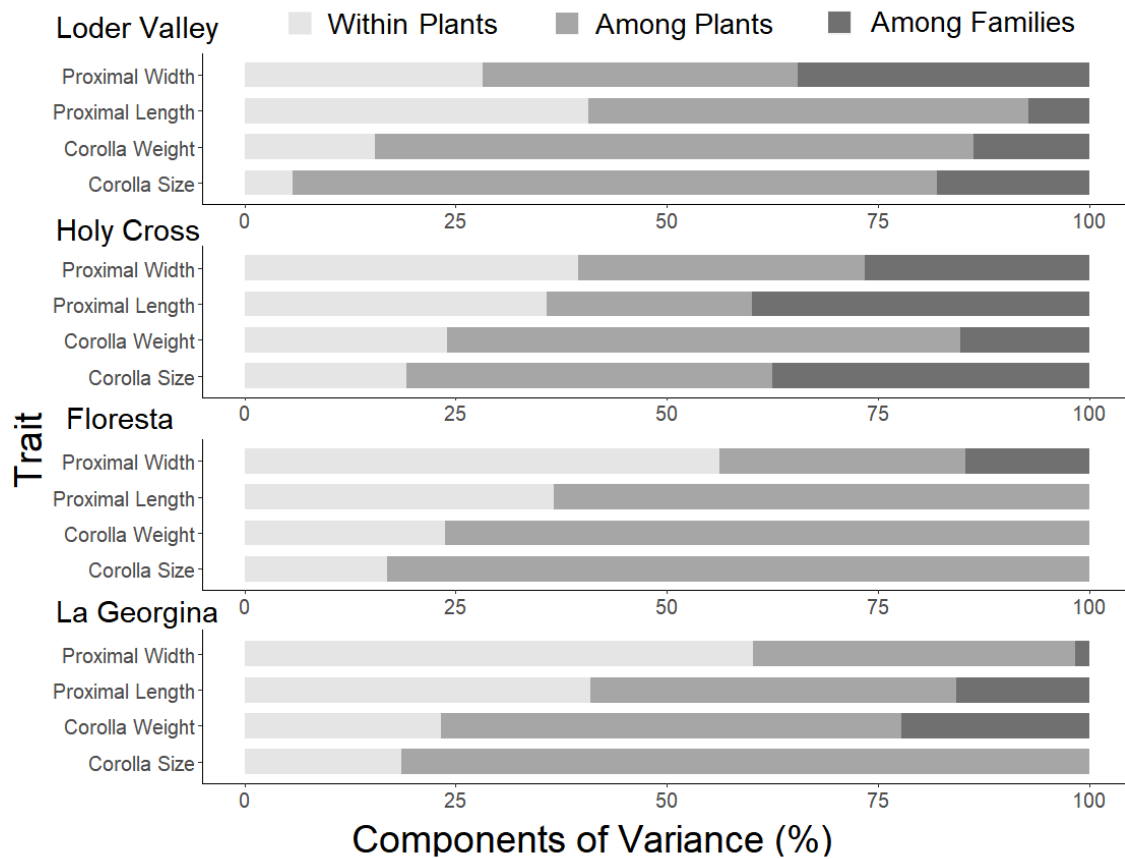
Figure 3.1. Components of variance for each floral trait, expressed as a percentage, among families (dark grey), among individuals within a family (grey) and among flowers within individuals (light grey) in plants from two populations in the native (Loder Valley and Holy Cross) and two from the introduced (Floresta and La Georgina) range.

3.3.3 Heritability Estimates

We found significant heritability for all traits, when tested across all populations included in the common garden. Parent offspring regression found a significant heritability for the whole corolla size (P = 0.013, h^2 = 0.44, N = 186; Fig. 3.3a) and the corolla weight (P = 0.047, h^2 = 0.27, N = 198; Fig. 3.3b). Plants also showed significant heritability for the proximal corolla length (P = 0.003, h^2 = 0.49, N = 191; Fig. 3.3c) and the proximal corolla width (P = 0.027, h^2 = 0.29, N = 191; Fig. 3.3d; Table 3.3).
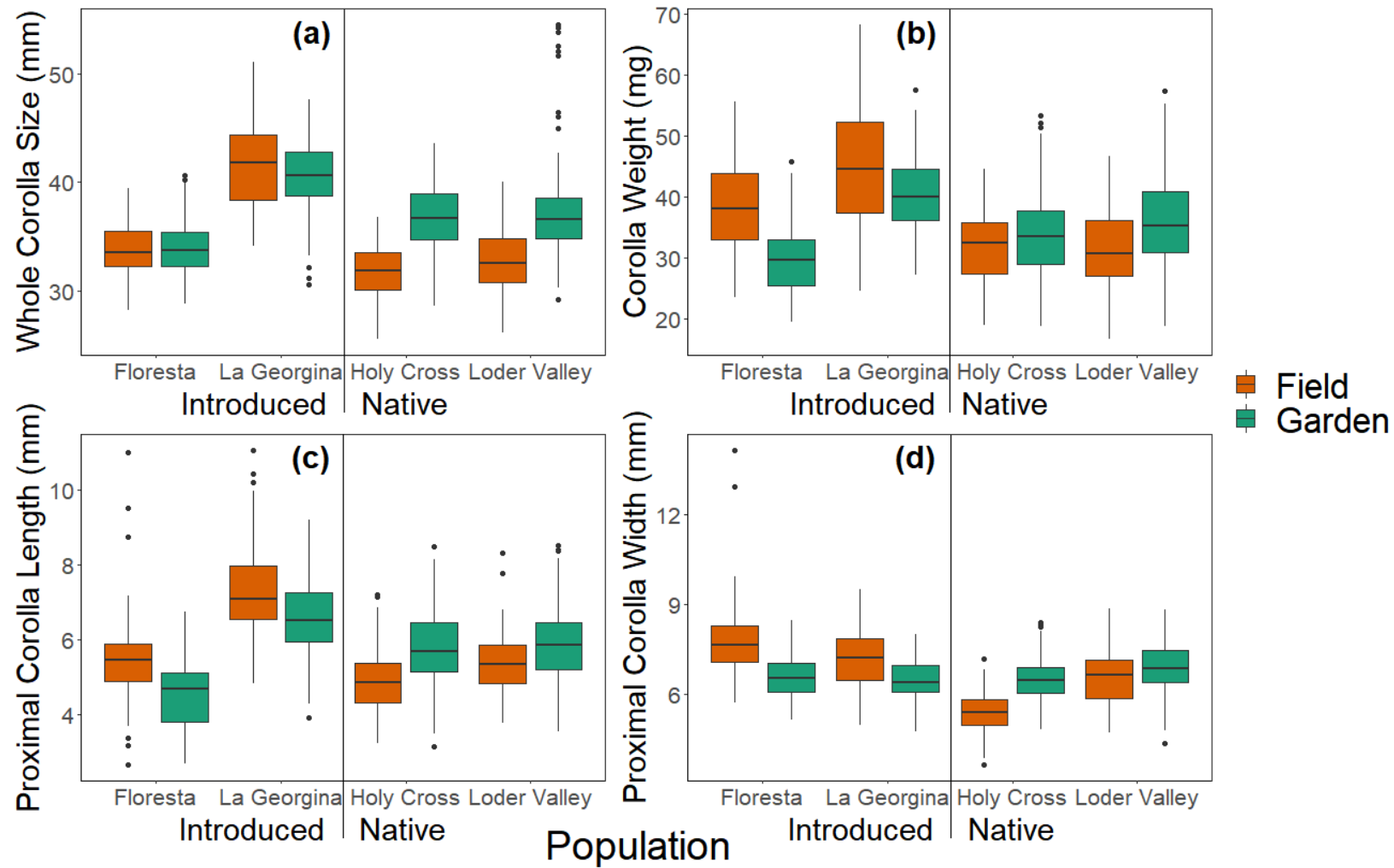
Figure 3.2. Comparison of corolla traits for plants sourced from native and introduced populations grown in the field (in orange) and the common garden (in green), for (a) whole corolla size; (b) corolla weight; (c) proximal corolla length and (d) proximal corolla width.

Table 3.3. Narrow sense heritabilities (h²) for floral traits in *Digitalis purpurea*. Included are the sample size (number of flowers), the slope, the 95% confidence interval of the slope, the heritability estimate and the P value. Significance of the regression is indicated by " " for P > 0.05; "*" for P < 0.05; "**" for 0.01 and "***" for 0.001.

Trait	Sample Size	Slope (b)	b 95% CI	h² estimate	P value
Whole Corolla Tube Size	186	0.2185	0.05 − 0.39	0.44	**0.013***
Corolla Weight	198	0.1338	0.01 − 0.27	0.27	**0.047***
Proximal Corolla Tube Length	191	0.2431	0.09 − 0.40	0.49	**0.003****
Proximal Corolla Tube Width	191	0.1461	0.00 − 0.27	0.29	**0.027***
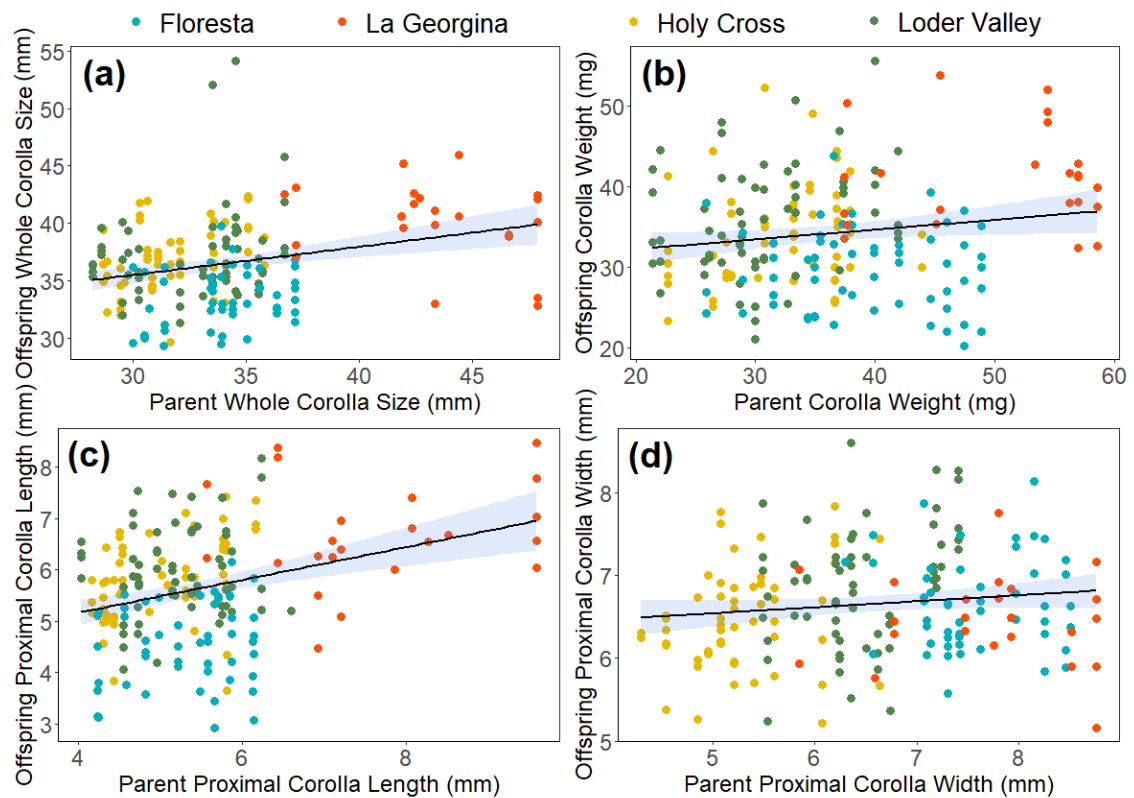
Figure 3.3. Parent-offspring regression of floral traits (N = 186 flowers) including (a) whole corolla size; (b) corolla weight; (c) proximal corolla length and (d) proximal corolla width. The population of origin is shown in the key above.

3.4. Discussion

For native and introduced populations of *Digitalis purpurea*, we find a strong genetic basis for floral traits with evidence from the high estimates of heritabilities. Additionally, trait means were higher in the garden for plants sourced from Britain but the opposite pattern for plants

sourced from introduced populations, indicating potential local adaptation to the environmental conditions of their populations of origin. Trait variation tended to cluster closer together around a common mean for plants grown in conditions of the common garden as opposed to the field conditions.

The floral traits showed significant and high narrow sense heritabilities across the common garden populations, with values ranging from 0.27 to 0.49. Our finding of a high heritability for the size of the corolla tube (h^2 = 0.44) is consistent with other studies that find the heritabilities of corolla length in the range between 0.14 and 0.77 (N =15 estimates from 9 species from 10 studies; shown in a review of h^2 estimates from studies in Appendix Table A3.2) and corolla width ranging from 0.16 to 0.66 (N = 15 estimates from 5 species from 6 studies; Appendix Table A3.2). With no studies to date investigating the heritability of the proximal corolla tube traits of a species, we can instead compare our results to the above ranges for other corolla traits since the proximal corolla tube in *D. purpurea* is functionally equivalent to the corolla tube in many other species. Through this, we find that our heritabilities for proximal corolla tube length (h^2 = 0.49) and proximal corolla tube width (h^2 = 0.29) are within the range for corolla traits (0.14 to 0.77; Appendix Table A3.2) and expectedly high considering the role of this trait in pollinator fit. The high heritability, combined with a high strength of selection previously found for this trait in introduced populations (selection coefficients of up to 0.32; see Chapter 2) could indicate a large directional change in trait size between generations, which could still be occurring for these traits to further accommodate pollinator morphology. Further work would be needed to confirm this, potentially through focussing on single field populations in the introduced range and calculating the expected evolutionary change by measuring the heritability and strength of selection on proximal corolla tube traits.

Calculating heritability with parent offspring regression holds several advantages compared with other methods, such as allowing an estimation of heritability from an incomplete pedigree. However there are also issues with this method, which include not being able to account for other sources of variation such as controlling for maternal effects (Lynch & Walsh, 1998). Our study also compares parents and offspring that developed in different environmental conditions, through which it is possible to overestimate the heritability (Conner et al., 2003; Kaczorowsi et al., 2008) but not in all cases (Young et al., 1994). In our common garden the groups sourced from each population had limited sample sizes (only up to 62 offspring for each population to compare with 15 parents), and small sample sizes might limit our ability to estimate accurate heritability values. Combining the populations allowed us to run the regression with enough power (N = 186) to calculate heritability. Considering these limitations,

this approach allows us an initial estimation of the heritability of the floral traits in the contexts analysed. The evidence of high heritabilites is further supported by our findings of large amounts of variation between families in most traits within populations (Fig. 3.1) and an additional method to calculate heritabilities of linear corolla traits using molecular markers indicate high and significant narrow sense heritabilities for *D. purpurea* in a similar range to those found in this study (Castellanos et al., Unpublished).

Along with a significant additive genetic underpinning to the variation, we find moderate environmental influence on the floral trait variation, shown by plants from British populations being brought into the common garden tending to express high trait values with variation distributed more closely around a global mean than in field populations (Fig. 3.2). The higher trait means could be caused by more adequate water availability, light levels and nutrient availability in the soil provided in a garden setting, which would allow plants to express higher phenotypic values than plants in the field (Alpert & Simms, 2002; Descamps et al., 2020; Galen, 1999; Poorter et al., 2012). To find the limits of plasticity in floral traits of *D. purpurea*, additional work could test how the floral trait variation responds to extreme environmental conditions, such as increased shade or decreased water availability (Gallagher & Campbell, 2017; Leibman et al., 2018). As with any sampling of a larger population, this can lead to founder effects such as a biased or unrepresentative subset of the larger population. However, our common garden experiment plants were chosen from a pool of parents that display the breadth of the floral trait variation, and are highly representative of that.

In contrast to the native British populations, we found that plants sourced from introduced populations from tropical mountains (Floresta and La Georgina) showed lower trait means when grown in the common garden located in the temperate native range. Whilst plants sourced from Britain may be better adapted to conditions of the southern England-based common garden (e.g. temperature, precipitation, herbivory or disease) that allow them to flourish (Joshi et al., 2001; Kawecki & Ebert, 2004), plants from the introduced populations may have lost local adaptation to conditions in southern England thus rendering conditions in Britain sub-optimal for growth (Williams et al., 2008). Whilst common garden experiments provide an effective way to study ecotypic differentiation and local adaptation in plants (Lortie & Hierro, 2021) they only provide a partial measurement of local adaptation (Nuismer & Gandon, 2008) with our study assuming a strong correlation between performance and expression of larger traits. Whilst our study uses floral trait sizes as a reflection of success based on this assumption, future work could estimate reproductive success as a measure of performance and compare between common garden and field populations. This study provides an estimation of the differentiation in plants

between ranges and opens a possibility for future work to test for local adaptation of plants in introduced and native parts of the range with equivalent garden experiments in the introduced range and a reciprocal transplant experiment (Oduor et al., 2016).

3.4.1 Concluding Remarks

We found high heritabilities for plant floral traits related to pollination success, since the recent finding of rapid evolution of two of those traits in introduced populations. Our finding of high genetic heritability of floral traits could indicate that the traits are still responding to the strong selection found in the introduced populations (see Chapter 2) and that the traits are still undergoing rapid evolution. Beyond testing this, it would also be worth further exploring our finding of reduced performance of plants from the tropical range in the southern England-based common garden, and whether differences in trait means are caused by differences in climate or adaptation to insect pests and diseases.

Chapter 4

Effects of novel nectar robbing on reproduction in *Digitalis purpurea*

Abstract

With many plant-pollinator interactions undergoing change as species' distributions shift, we require a better understanding of how the addition of new interacting partners can affect plant reproduction. One such group of floral visitors, nectar robbers, can deplete plants of nectar rewards without contributing to pollination. The addition of nectar robbing to the floral visitor assemblage could therefore have costs to the plant´s reproductive output. We focus on a recent plant colonist, *Digitalis purpurea*, a plant that in its native range is rarely robbed, but experiences intense nectar robbing in areas it has been introduced to. Here, we estimate the costs to reproduction following experimental nectar robbing. To identify any changes in the behaviour of the principal pollinators in response to nectar robbing, we measured visitation rates, visit duration, proportion of flowers visited and rate of rejection of inflorescences. To find the effects of robbing on fitness, we used proxies for female and male components of reproductive output, by measuring the seeds produced per fruit and the pollen export respectively. Nectar robbing significantly reduced the rate of visitation and lengths of visits by bumblebees. Additionally, bumblebees visited a lower proportion of flowers on an inflorescence that had robbed flowers. We found that flowers in the robbed treatment produced significantly fewer seeds per fruit on average but did not export fewer pollen grains. Our finding that robbing leads to reduced seed production could be due to fewer and shorter visits to flowers leading to less effective pollination. We discuss the potential consequences of new pollinator environments, such as exposure to nectar robbing, for plant reproduction.

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4.1 Introduction

Many plant-pollinator interactions are undergoing change due to multiple anthropogenic influences (González-Varo et al., 2013, Goulson et al., 2015). At present we have a limited understanding of how novel interactions affect plant reproductive success, for example, after plant invasion (Barrett et al., 2008; Chalcoff et al., 2019; Richardson et al., 2000). One interaction with consequences for plants is nectar robbing, where plants have their mutualism with pollinators bypassed by floral visitors (robbers) that consume nectar rewards without pollinating (Irwin et al., 2010). In the same way as pollination mutualisms, this interaction between plants and nectar robbers can also be altered as a consequence of changes in abundance or distributions of the plants or floral visitors (Irwin & Maloof, 2002; Traveset et al., 1998). In turn, changes in the incidence of nectar robbing have the potential to affect a plant´s reproductive success.

During nectar robbing, a floral visitor bites a hole in the corolla ("primary robbing") or utilises an existing hole previously created by another robber ("secondary robbing") to feed from nectar, which often results in no contact with the stigmas or anthers and hence no contribution to pollination (Inouye, 1983; Rojas-Nossa et al., 2016). Some previous studies found limited or no negative fitness consequences of robbing for the plant (Richman et al., 2018; Stout et al., 2000) with some examples of robbing increasing plant fitness through increasing pollen flow and dispersal distance (Higashi et al., 1988; Maloof & Inouye, 2000) and increasing the frequency of visitation from legitimate pollinators (Stout et al., 2000). However, other studies have reported detrimental effects on at least one component of the plant's reproductive success (Adler et al., 2016; Burkle et al., 2007; Castro et al., 2008; Irwin & Brody, 1999; Lara & Ornelas, 2001;). Negative effects of robbers include damage to the reproductive organs, a reduction of the attractiveness of the floral display, and exhaustion of the nectar reward, all of which could potentially alter the foraging behaviour of legitimate pollinators that are required for plant reproductive success (Irwin et al., 2010). The extent to which robbers affect plant fitness could depend on the frequency, the amount of damage done, and how much the behaviours of the legitimate pollinators are affected (Adler et al., 2016; Irwin et al., 2010). Additionally, if extra resources are allocated towards nectar production in the plant in response to robbing, this could have a detrimental effect on the number of seeds and/or fruits produced (Navarro, 2001; Pyke, 1991; Southwick, 1984). In this study we focus on the effects of robbing on a plant that, after

range expansion, experiences a high level of nectar robbing that is not present in the native range.

The common foxglove, bumblebee-pollinated *Digitalis purpurea* L. (Plantaginaceae)*,* expanded its range from native European woodland to areas including tropical mountains in Central and South America following anthropogenic introductions (Mackin et al., 2021; see Chapter 2). As a consequence, the plant now experiences geographically variable rates of nectar robbing. The pollination biology of this species is well known in the native range, yet to our knowledge, only one record exists of nectar robbing in populations across Europe, associated with robbing specialist *Bombus wurflenii* (Reinig & Rasmont, 1988), whereas in American populations we found that the plants are robbed at a high rate (Fig. 4.1). For example, in preliminary observations in 110 plants across two non-native populations in Colombia, we recorded that 288 out of 677 (42.5%) recently opened flowers had been robbed at least once (pers. obs.). In these populations, the bumblebees *Bombus hortulanus* and *B. rubicundus,* and additionally some species of hummingbird and flower piercers (specialised robbers in the genus *Diglossa*), frequently feed on nectar from *D. purpurea* by robbing the flowers. In Costa Rica, the high-altitude bumblebee *B. ephippiatus* is the main pollinator of the plant but also a frequent robber. Frequently, individual bumblebees use a mixed foraging strategy on *D. purpurea*, robbing and visiting legitimately on the same foraging bout.

Here we test the costs of novel levels of nectar robbing on *D. purpurea* by experimentally robbing plants and measuring the effect on pollinator behaviour and on the plant's reproductive success. We expect nectar robbing will reduce the volume of nectar or alter the rate of nectar production, causing bees to visit flowers at a lower rate (Parachnowitsch et al., 2019). Additionally, it is possible that, through increased replenishment of nectar, robbing could also reduce energetic resources available for fruit production, regardless of effects on bee visitation (Navarro, 2001). We measured both male and female components of reproduction for plants and quantified the visitation rates by naturally foraging bumblebees to each treatment. We also recorded other measures of bumblebee visitation patterns on inflorescences, including time spent visiting flowers and proportion of an inflorescence's flowers that were visited in a foraging bout.

4.2 Materials and Methods

4.2.1 Study system and experimental set-up

The facultative biennial herb *D. purpurea* (Plantaginaceae) produces long showy inflorescences (Fig. 4.1) of nectar-rich flowers that open in sequence from the bottom up. The flowers are protandrous, with anthers dehiscing following anthesis and the stigma becoming receptive three to five days after anthesis. Animal pollination is required to achieve full seed set (Mackin et al., 2021; Nazir et al., 2008; also see Chapter 2), and the primary pollinators in the native range are the long-tongued bumblebees *Bombus hortorum* and *B. pascuorum* (Grindeland et al., 2005; Broadbent & Bourke, 2012). In introduced populations in Central and South America, hummingbirds are also important pollinators, but several species of *Bombus* remain the most frequent ones (Mackin et al., 2021; also see Chapter 2). In those populations we also observe high levels of nectar robbing by bumblebees (Riveros et al., 2006), *Diglossa* flower piercers, and hummingbirds. We have not observed bumblebees making holes on the corollas; instead, bees and hummingbirds are likely secondary robbers using the holes pierced by *Diglossa* birds. In surveys in two localities in Colombia (Floresta N = 50 and Choachí N = 60) we found that 36.2% and 47.8% (respectively) of all recently opened flowers were robbed, with robbing making up to 14.1% to 19.4% of all visits to flowers in those two populations.

Nectar production and secretion begins the day before the first pair of anthers dehisce, and peaks during anthesis and stigma maturation (Percival & Morgan, 1965). Nectar is produced in floral nectaries located at the base of the ovary and escapes through modified stomata that are permanently open (Gaffal et al., 1998). Nectar sugar concentration ranges from 16-27% and is predominately made up of sucrose (78.5%) with some glucose and fructose sugars (Gaffal et al., 1998). In our study populations, a single flower secretes between 3.1 to 10.5µl of nectar over 24 hours without visitation (pers. obs.).

We grew *D. purpurea* potted plants from seed collected from a wild population near Portsmouth (Britain) in 2017, that were grown until flowering in summer 2019 and then transported to the University of Sussex campus in Falmer. Between 16-18 plants were selected at random to be in one of two treatment groups – "robbed" and "control" (non-robbed flowers). To standardise the rate of natural pollination that all plants experienced, plants were exposed for 3 hours each day to receive visits by local bumblebees, and for the other period of 21 hours the inflorescences were covered with a mesh bag to block visitation.

Plants in the robbed treatment group had all recently opened flowers manually robbed by piercing a hole in the proximal corolla tube with a microcapillary tube twice during the 3-hour

pollination period (once at the start, and then again after 90 minutes). This rate of robbing is based on field observations in the non-native range where *D. purpurea* flowers are robbed less than once per hour, and on greenhouse trials on the experimental plants that showed that nectar is replenished at a rate of 2.3μl per hour during three hours following nectar depletion (N = 30 flowers over 10 plants). Compared to other species tested, this is a fast rate of replenishment (Castellanos et al., 2002) as a single foxglove flower secretes up to 10.5μl of nectar per day.

Bumblebees will often avoid plants that have recently been visited by other floral visitors using olfactory and other cues (Stout et al., 2000), so we manually handled the inflorescences (both controls and robbed) to mimic contact during simulated robbing. This entire procedure was repeated daily for the period that plants flowered between June 17 and July 3, 2019.

Figure 4.1. *Bombus hortulanus* worker robbing nectar from a hole at the base of *Digitalis purpurea* flowers in a non-native population in Colombia. Photo by MC Castellanos.

4.2.2 Bee behaviour and Visitation Rates

Apart from the robbing behaviour, *Bombus* species in the native and non-native ranges behave very similarly when visiting foxgloves flowers. In this experiment, we focus on native

bumblebees to understand how robbing affect bumblebee pollination in general. To test if robbing leads to altered behaviour of bumblebees, we recorded several aspects of visitation. Firstly, we tested whether robbing would cause a change in visitation rates, by quantifying the number of visits per flower per hour on plants in both treatments when in full bloom. We also recorded the identity of bumblebee species that visited when conducting censuses. Plants were arranged in a line, separated by about 1 meter, with alternating treatments and the individuals within each treatment had their positions randomised. We recorded counts of each bumblebee species visiting flowers on control and robbed treatment plants and used 644 3-minute censuses to quantify visitation rates in 3-hour periods that covered different times of the day when floral visitors were active. Visitation rates (visits per flower per hour) were compared between robbed and non-robbed plants using generalised linear models with a binomial distribution (flowers visited vs not visited) in the *stats* package in R (R Core Team, 2020). We included the pollinator species as a fixed factor in the model to test for differences between the two bumblebee species, and a term for the interaction between treatment and pollinator.

Secondly, we also measured potential effects of robbing on the duration of floral visits by bumblebees. We recorded with a stopwatch the length of visits to individual flowers, defined as the time between entering a flower to feed and appearing at the mouth of the corolla to exit it. The duration of a visit to a flower was compared between robbed and non-robbed plants using linear models in R, where visit duration was log-transformed as the data showed a skewed distribution. We included pollinator as a fixed variable in the model, to test for differences in visitation by the two species.

Bumblebees typically visit several flowers on a foxglove inflorescence in each foraging bout, so to test whether robbing had an effect on a continuous foraging bout we measured the proportion of flowers on an inflorescence that individual bumblebees visited per foraging bout. Finally, we also recorded the number of times a plant in a treatment was 'rejected', defined as a bumblebee hovering near flowers and leaving without landing, or landing on the flower and leaving without entering. We tested for differences between control and robbed treatment plants for the proportions of flowers visited (with pollinator species included as a fixed factor) and the proportion of rejections using generalised linear models with a binomial distribution in the *stats* package in R.

4.2.3 Effects on reproductive success

To test whether plant reproductive success was impacted as a result of nectar robbing, we estimated male and female components of reproductive output. For this we measured the pollen export and number of seeds produced on average by experimental plants, then compared between treatments to test for differences between robbed plants and control plants.

4.2.4 Pollen Export

We used pollen export as a proxy for the male component of reproductive success, by quantifying the number of pollen grains removed from an anther by visitors in a 3-hour period. In *D. purpurea* flowers, two anterior anthers are paired with each other and tend to dehisce simultaneously (Percival & Morgan 1965), as do the two posterior anthers further inside the flower. To find the number of pollen grains removed (= pollen export), we subtracted the number of pollen grains left on a "post-visitation" anther from the number on a "pre-visitation anther" on the same flower. For this, immediately before the beginning of each allotted visitation period (whereupon inflorescences are still bagged), we collected one of the near-dehisced posterior anthers (the "pre-visitation" control anther) in a centrifuge tube. Once dehisced in the tube, we added 70% ethanol until counting. We collected the corresponding anterior "post-visitation" anther (which was already dehisced before the visitation period begun) from the same flower immediately after the visitation period of 3 hours and stored it in 70% ethanol. We did not record the number of visits to "post-visitation" anthers, but each flower is likely to have received multiple visits. This was repeated for each plant on separate days to include 2-6 anther comparisons per plant for at least 12 plants per treatment.

We used a haemocytometer counting chamber to estimate the number of pollen grains on the "pre-visitation" and "post-visitation" anthers. Up to 30 minutes prior to counting, tubes containing anthers were sonicated for four lots of 30 seconds to dislodge pollen grains and to homogenise the grains in the ethanol. Immediately before counting, the ethanol-pollen mixture was then vortexed for five seconds and 8µl of the sample was pipetted into the chamber for counting. Pollen grains were counted in grid squares of a known volume (0.1 µL) and multiplied up for an estimate of the total number of pollen grains in the original 200µl sample. We repeated this process four times for each sample and averaged for analysis. We calculated total pollen export by subtracting the number of pollen grains estimated to be left on a post-visitation anther

from the number of pollen grains estimated to be on the corresponding paired pre-foraging anther.

This method assumes that the number of grains in anthers within a flower is similar. To test this, we counted the number of grains in all four anthers of eight flowers from different individual plants using the same method as above. We found that the anterior anthers did not produce significantly different numbers of pollen grains to the posterior anthers in a flower (P = 0.451; N = 32 anthers). This confirms that anthers in a given flower can serve as good pre-visitation controls in our experiment.

We compared pollen export between the robbed and control treatments using mixed effects linear models in R, with plant individual as a random factor (packages *lme4* and *lmerTest,* Bates et al., 2015).

4.2.5 Seed production

We used seed production per fruit as a proxy for the female component of plant reproductive success. Flowers on inflorescences received a daily 3-hour 'window' of pollination by natural bumblebee visitation for the entirety of their flowering period. After the flowering had finished, we left plants unbagged to allow fruits to develop normally. Around 4-6 weeks after flowering, we collected between three and seven near ripe but undehisced fruits from each plant at various points on the inflorescence and then left them to dry inside paper envelopes to allow natural dehiscence of fruits. We photographed seeds on filter paper and then counted using a macro in ImageJ software.

We compared seed production between the robbed and control treatments using mixed effects linear models in R, with plant individual as a random factor (packages *lme4* and *lmerTest,* Bates et al., 2015).

4.3 Results

4.3.1 Bee behaviour and Visitation Rates

As expected, two bumblebee species were the only pollinator visitors to our experimental plants, with *Bombus hortorum* visiting flowers significantly more often (mean = 0.6 ± 1.6 SD visits

per flower per hour) than *B. pascuorum* (mean = 0.2 ± 0.9 SD; P < 0.001; N = 1288 3-minute surveys). Overall, robbed flowers received visits at a significantly lower rate (mean = 0.7 ± 1.7 SD visits per flower per hour) than flowers in the control treatment (mean = 1.0 ± 2.0 SD visits per flower per hour; P < 0.001; Fig. 4.2a), and this was consistent for both bumblebee species. *B. hortorum* visited control flowers and robbed flowers at rates of 0.7 ± 1.7 SD and 0.6 ± 1.5 SD visits per flower per hour (respectively) whilst *B. pascuorum* visits control and robbed flowers at rates of 0.3 ± 1.0 SD and 0.2 ± 0.7 SD visits per flower per hour (respectively).

We found that the average visit length to flowers was also different when comparing robbing treatments. Visits to robbed flowers were significantly shorter (mean = 6.6 ± 5.3 SD seconds; N = 120) than on the control flowers (mean = 10.3 ± 9.5 SD seconds; N = 210; P < 0.001), with visits being on average 3.7 seconds shorter (Fig. 4.2b). This reduction in visit length was consistent across bumblebee species (Fig. 4.3); however, visits by *B. hortorum* were overall of shorter duration (mean = 7.3 ± 6.3 SD seconds; N = 232) than *B. pascuorum* (mean = 12.9 ± 11.2 SD seconds; N = 98; P < 0.001; Fig. 4.3).

Bumblebees visited on average 50% of a control plant's flowers on an inflorescence (N = 52) and 42% of a robbed plant's flowers (N = 40), and this difference was statistically significant (P = 0.037). When recording the number of times that plants in each treatment were rejected by foragers, robbed plants were rejected 32 times compared with 25 times for the control; this was not significantly different (P = 0.090).
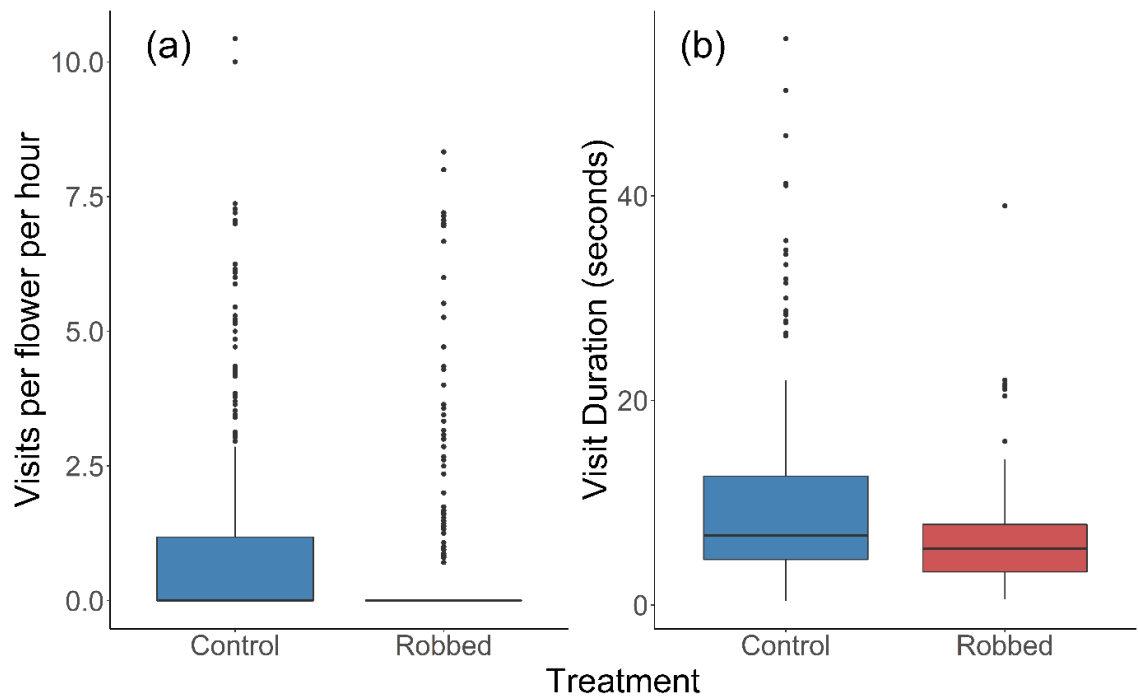
Figure 4.2. (a) The mean number of visits per hour to flowers by all bumblebees to non-robbed control (blue, N = 322) and robbed plants (red, N = 318). (b) The mean flower visit duration by all bumblebees to non-robbed control (blue, N = 210) and robbed plants (red, N = 120)
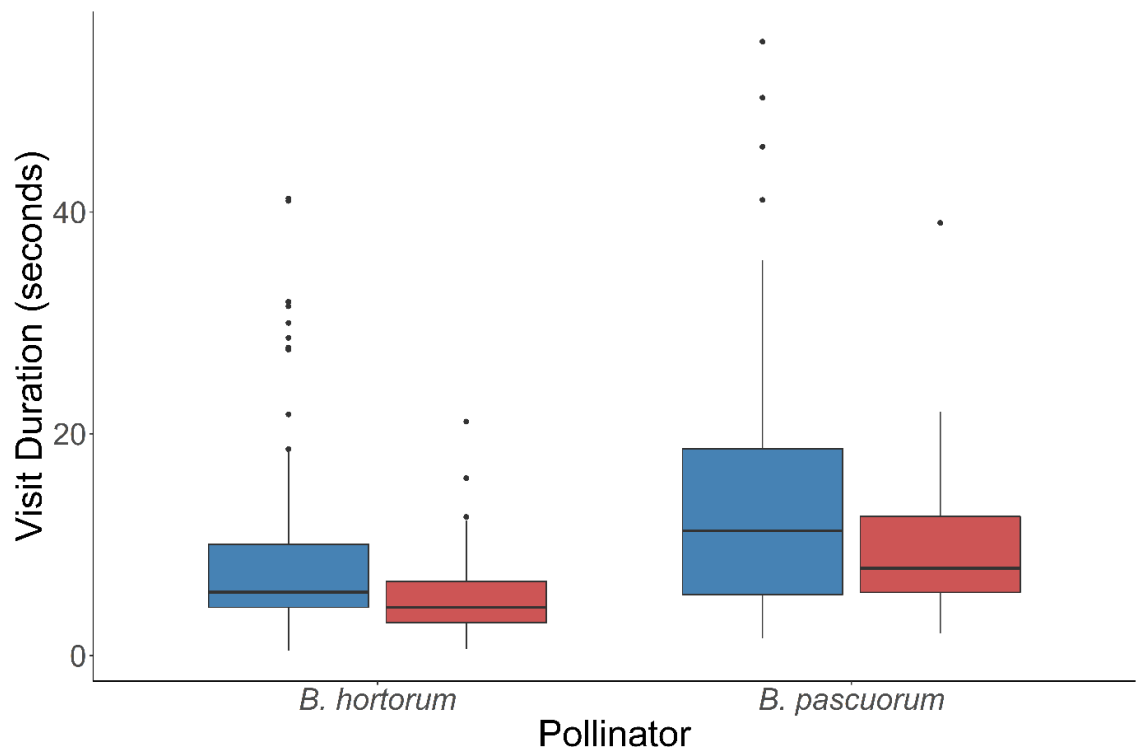
Figure 4.3. The mean visit duration to flowers by *Bombus hortorum* (N = 147) and *B. pascuorum* (N = 63) to non-robbed control (blue) and robbed (red) plants.
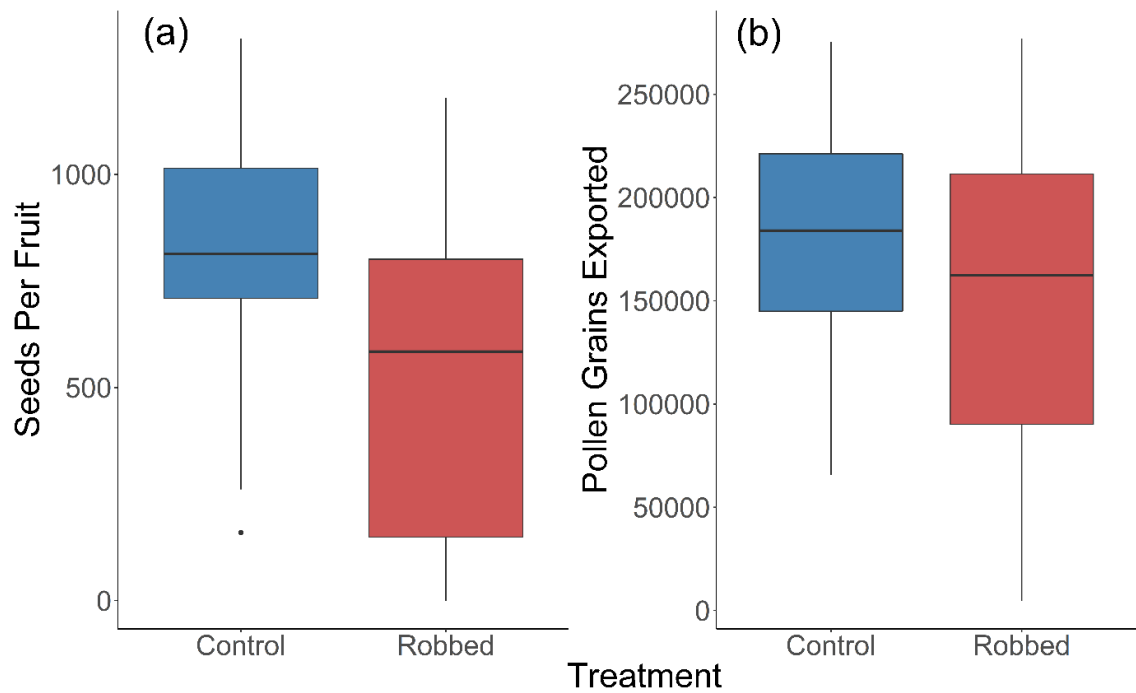
Figure 4.4. (a) The mean number of seeds produced per fruit in the non-robbed control (blue, N = 85) and robbed plants (red, N = 59). (b) Number of pollen grains exported from anthers in non-robbed control (blue, N = 43) and robbed (red, N = 50) plants.

4.3.2 Effects on reproductive success

Fruits in control plants produced significantly more seeds (mean = 823.6 ± 48.98 SD seeds; N = 87 fruits) than those in robbed plants (mean = 510.7 ± 71.18 SD seeds; N = 72; P < 0.001; Fig. 4.4a), with robbed plants producing 25% fewer seeds on average compared with control plants. In contrast, export of pollen grains was not significantly different between the robbed (149,167 ± 72,742 SD pollen grains, N = 50) and the control (178,329 ± 57,904 SD pollen grains, N = 43) treatments (P = 0.141; Fig. 4.4b).

4.4 Discussion

With our experimental approach we show how the addition of high levels of nectar robbing can have a cost to the reproductive output of a plant colonising a new area. This cost is in terms of seed production, with robbed plants producing 25% fewer seeds per fruit compared with non-robbed controls. Below we discuss how this result can be related to changes in pollinator behaviour and the potential implications of these costs for the naturalised populations exposed to novel levels of robbing.

Visiting bumblebees altered their behaviour when interacting with robbed plants by having a significantly reduced visit rate and visiting a smaller proportion of flowers in the inflorescence compared to non-robbed plants. This is consistent with other experimental studies removing nectar (Li et al., 2021) and with the fact that bumblebees tend to spend less time on an unrewarding inflorescence, where departure can be triggered by encountering one or more unrewarding flowers (Best & Bierzychudek, 1982; Heinrich, 1979). We also found that bumblebees reduced the length of floral visits on robbed flowers compared to non-robbed flowers. This could potentially be due to a lower volume of nectar present in robbed flowers taking less time to drink and bumblebees leaving sooner (Hodges & Wolf, 1981). Richardson (2004) also found that bumblebee visit duration was reduced in robbed flowers compared with unrobbed flowers. Other studies have found that robbing is associated with reduced visitation rates (Irwin et al., 2010), and some suggest that robbing holes and damage to the flower is off-putting to visitors (Goulson et al., 2007; Varma et al., 2020; although this is not always the case (Souza et al., 2019)). In our experiment (as in non-native field populations of *D. purpurea*) holes were made at the base of the corolla, which in field conditions may be out of visual range for legitimate visitors to detect and be repelled by, especially for the fast paced foraging of hummingbird pollinators. However, since there is evidence that hummingbirds can use visual cues to discriminate against robbed flowers (Lara & Ornelas, 2001), it would be interesting to assess to what degree hummingbird pollinators in the introduced range are dissuaded from pollinating by the robbing holes as well as from a lack of nectar resulting from robbing (Irwin, 2000). In addition, future work on this system could add a control to test the effect of a visitor piercing the corolla to make robbing holes.

The two visitors to our experimental plants were *Bombus hortorum* and *B. pascourum*, with the former visiting three times more often, consistent with what is observed in nature in Britain (Broadbent & Bourke, 2012; Mackin et al., 2021). Interestingly, *B. pascuorum* visited individual flowers for a longer duration, regardless of robbing treatment. This could be explained in part by the shorter tongue length of *B. pascuorum*, causing them difficulty feeding in *D. purpurea* flowers and so they take a longer time to complete a visit. In any case, both bumblebee species showed the same patterns of reduced visitation to robbed flowers compared to non-robbed ones.

The change in bumblebee behaviour could be contributing to the lower reproductive output we find for the female component of reproduction in robbed plants, with intense levels of nectar robbing causing less frequent and shorter visits which ultimately reduces pollen deposition and therefore seed production. This idea is supported by several studies that find the duration of

visits by bumblebees positively correlates with pollen deposition (Cresswell, 2000; Kudo, 2003; Thøstesen & Olesen, 1996). This is not always the case; in a study by Richardson (2004) bumblebees spent less time in robbed flowers but visit duration did not correlate with amount of pollen deposited. Other authors finding similar results to ours (Irwin & Brody, 1999; Lara & Ornelas, 2001), suggest that reduced attractiveness of flowers can lead to a reduction in the pollinator visitation rate and a lower seed production. However, we cannot rule out the possibility that resource depletion resulting from manual robbing also contributed to the reduced seed production. This could be caused by increased metabolic costs for the plants associated with increased production of nectar (Doust & Doust, 1988; Navarro, 2001). Future work on robbing could use pollen supplementation to distinguish between the effects of changes in nectar robber behaviour from metabolic costs of nectar on reproductive output.

In contrast to findings we present here, many studies suggest robbing can have limited or no negative effects on the female component of fitness (Andalo et al., 2019; Carrió & Güemes, 2019; dos Santos et al., 2020; Maloof, 2001; Richardson, 2004; Varma et al., 2020; Varma & Sinu, 2019; Zimmerman & Cook, 1985). This lack of an effect on reproductive output could be due to the legitimate pollinators still visiting the plant and saturating the stigmas with enough pollen so the plant can achieve full seed set (Heiling et al., 2018; Stout et al., 2000). One potential caveat in this study is that our ability to detect differences in mean seed production could be biased by the standardised three-hour visitation periods per day in our experiment. This is because open visitation during the lifetime of the flower could lead to full seed set, even with a reduced visitation rate. However, even with this restriction on the amount of visits plants could receive, these experimental results are consistent with the comparatively low seed set we have observed in the non-native populations (as we discuss below).

We found that nectar robbing did not negatively affect the male component of reproduction through pollen removal by bumblebees. Other studies found that nectar robbing can include a cost to the male component of fitness in some species (Castro et al., 2008; Irwin & Brody, 1999; Irwin & Maloof, 2002; Richardson, 2004) but not in others (Maloof, 2001; Morris, 1996; Richman et al., 2018). With the method we used here, it is unclear how much of the pollen released from anthers ultimately reaches stigmas of conspecific plants. As with using any proxy as a measure of reproductive success, in this case it is difficult to deduct the entire picture as to whether nectar robbing affects male success.

The aim of our experiment was to simulate the conditions of nectar robbing on potted plants of *D. purpurea*, with the idea that similar effects could be found for plants in the non-native robbed

field populations. The reduced seed production following addition of nectar robbing we observe here is consistent with our previous observations showing that non-native plants in populations with nectar robbers have a significantly lower lifetime seed production (average = 40,788 ± 20,644 SD seeds, across three populations in Colombia and Costa Rica; N = 211 plants) compared with native populations with no robbing (average = 113,812 ± 84,868 SD seeds across two populations in Britain; P < 0.001; Appendix Fig. A4.1; see also Chapter 2). Although many other factors could be involved, the high levels of nectar robbing could be contributing to the lower average reproductive output in the introduced range. In pollinator surveys in the same naturalised populations in Colombia we found that individual *Bombus hortulanus* and *B. rubicundus* bumblebees used a mixed strategy of visiting flowers both legitimately and robbing. This can be common in robbing interactions (Morris, 1996) although often bumblebees adhere to a consistent strategy to reduce handling time during a foraging bout (Bronstein et al., 2017). If plants are already receiving adequate pollination, then nectar robbing may only incur reproductive costs if a certain threshold of robbing intensity is reached that depletes nectar or alter visual cues enough to deter legitimate visitors (Irwin et al., 2015).

With the intensity of nectar robbing varying across populations so radically, there could be considerable differences among populations in robber-mediated selection on floral traits (Castro et al., 2008; Navarro & Medel, 2009). Plant populations experiencing a high level of robbing could evolve local resistance or tolerance to nectar robbing (such as phenological, mechanical or chemical barriers) even at the cost of decreasing the attraction to pollinators and reducing reproductive output compared with other populations (Adler et al., 2016). It is intriguing that native *D. purpurea* populations experience low levels of nectar robbing, even in the presence of bumblebee species that are capable of making holes and often rob other plant species (*Bombus terrestris, B. lucorum and B. wurflenii*). *D. purpurea* plants produce high levels of toxic cardenolide compounds (Evans & Cowley, 1972) that are also present in the nectar (Palmer-Young et al., 2019). It is possible that toxic compounds in foxglove nectar are differentially toxic to particular visiting species, for example generalist robbers, influencing whether they can feed on the plant as has been seen in other species (Barlow et al., 2017; Tiedeken et al., 2016; Villalona et al., 2020). Further work into the potential role of nectar toxicity and other floral traits and how their relationship with fitness changes under different intensities of nectar robbing in *D. purpurea* could give insight into how nectar robbing can affect the trajectory of a plant's evolution.

4.4.1 Concluding Remarks

Our findings contribute to the growing body of evidence that a changed pollination environment, including nectar robbing, can have strong effects on visitation to a plant and the subsequent reproductive output. The addition of novel floral visitors to a plant's assemblage is likely to become more frequent as plants and nectivorous animal ranges shift due to human influence (Cheptou et al., 2017; Valiente-Banuet & Verdú, 2013). Therefore, it is important to understand how plants are likely to respond or change as a result of addition of new interacting partners. Further studies on this system could examine whether the addition of nectar robbers affects reproductive output in natural populations and how different nectar robbing communities in different parts of the range of *D. purpurea* are affecting the plant's evolution.

Chapter 5

The relationship between selfing and floral traits in *Nicotiana glauca*

Abstract

With many plants and their pollinators currently undergoing range changes and declines, the instances of plants losing pollinators is increasing. Loss of animal pollination for a plant can result in reliance on self-pollination, but the mechanical aspects underpinning this shift in breeding system are not fully understood. To investigate the link between floral trait variation and selfing, we focussed on a widely distributed self-compatible species, *Nicotiana glauca*, a plant pollinated by hummingbirds in its native range that predominantly self-pollinates in non-native invasive populations that lack pollinators. Here, we test for a relationship between seed production by selfing and the variation of floral traits thought to be influencing fertilisation, including stigma-anther (SA) distance, and corolla length, width and flare size. In non-native field populations in Tenerife we tested for a relationship between floral traits and selfing at the level of the plant individual, whilst in potted plants in a common garden we tested for a relationship between selfing and SA distance at the level of the flower. An experiment on the breeding system confirmed that flowers in Tenerife rely on selfing for pollination. We also conducted surveys to check whether there were any floral visitors to the plant, and we did not observe pollinators in the daytime or evening. We found a significant negative and non-linear relationship between SA distance and the propensity of flowers to self when tested at the level of the flower, but we did not find this relationship at the level of the plant individual. This, along with the high levels of phenotypically plastic variation within plants, decreases the likelihood that there can be a rapid response to directional selection for shorter SA distances. This study contributes to an explanation of how plants can cope with pollinator loss through variation in floral traits and the mating system.

5.1 Introduction

Following loss of animal pollination, plants will often revert to self-pollination to achieve reproduction (Pannell, 2015). However, we need further work to understand how this reproductive strategy is controlled by floral morphology (Opedal, 2018). For example, hermaphroditic angiosperms often have male and female reproductive parts spatially separated (herkogamy) which can reduce the likelihood of self-pollination and the potential for inbreeding depression (Webb & Lloyd, 1986). Self-compatible plants with a high capacity for autonomous selfing often have short distances between their flowers' anthers and stigma compared to species that predominantly outcross (i.e. decreased herkogamy; Opedal, 2018), with pollen being released at a shorter distance from the stigma leading to flowers having a higher probability of self-fertilising (Kalisz et al., 1999). Other corolla traits, such as the width of the corolla, could also affect the probability of selfing (Dart et al., 2012) potentially through pushing reproductive parts closer together. Whilst there are many examples of the switch to predominantly selfing following pollinator loss (e.g. Opedal et al., 2016; Pannell, 2015; Petanidou et al., 2012; Razanajatovo et al., 2016), there are only a handful of studies on how individual variation in floral traits can influence the prevalence of selfing (Armbruster et al., 2002; Brunet & Eckert, 1998; Eckert & Schaefer, 1998; Issaly et al., 2020).

To study the direct relationship between floral traits and selfing we focused on a species that displays variation in both its breeding system and pollinator assemblages across its distribution. The tree tobacco, *Nicotiana glauca* (Solanaceae), is a self-compatible plant native to central and northwest Argentina and Bolivia, where it relies on hummingbird pollinators for outcrossing (Nattero & Cocucci, 2007; Nattero et al., 2011). In other parts of the Americas invasive populations are also hummingbird pollinated, whilst sunbirds and honeyeaters perform this role elsewhere in the world (Schueller, 2004, Ollerton et al., 2012). In the absence of suitable pollinating birds, other non-native populations around the globe rely on self-pollination (Ollerton et al., 2012). Previous work on *N. glauca* investigated how differentiation of the floral morphology correlates with the different pollinator assemblages that visit (García et al., 2020; Nattero & Cocucci, 2007; Nattero et al., 2010; Schueller, 2007). Synthesising the then-available

information, Ollerton et al., (2012) showed that mean stigma-anther distance reduced predictably by more than one third between native populations and non-native selfing populations, with non-native bird-pollinated populations intermediate between these two extremes. However there is enormous within- and between-population variability in stigma-anther distance. When breeding system changes in the presence or absence of pollinators were investigated by Issaly et al., (2020) they found that, at the level of the individual plant, stigma-anther distances did not have a relationship with selfing. Testing this relationship at the level of the flower could help us understand whether there is a direct relationship between the floral traits and selfing and hence, the mechanistic explanation for how this mode of reproduction comes to predominate in some self-compatible species.

Individuals of *N. glauca* can express a considerable amount of phenotypic variation in floral traits, such as size and curvature of the corolla (García et al., 2020). A large amount of within-individual variation could influence whether any relationship between floral traits and selfing is detected when testing using individual average values. Moreover, this would affect how the variation present among plant individuals can respond to selection for increased selfing (Whitman & Agrawal, 2009). Therefore, to go beyond testing for a relationship between floral traits and selfing in the field, in the present study we utilise a common garden setting to directly relate the effect of a stigma-anther distance to the reproductive output of the same flower. Then, by comparing this with our test for a relationship at the level of the plant, we can explore the implications of this for the breeding system and evolutionary trajectory of the plant.

In this study we focus on *N. glauca* to investigate how selfing can be influenced by variation in floral traits at the individual plant and floral levels. We studied plants in non-native field populations to examine the relationship at the individual level, and used plants grown in a common garden with seed sourced from field populations to study the relationship at the level of the flower. We expect that stigma-anther distance (here on referred to as SA distance), corolla width, corolla length and corolla flare could play a role in the rate of selfing. Our hypothesis is that there will be a negative relationship between SA distance and selfing, whereupon as the SA distances become narrower, more seed is set (i.e. greater selfing) since with greater proximity there is a greater probability of pollen transfer from anthers to stigma. We also expect that one or a combination of the traits of corolla length, width and flare could show a negative relationship with selfing, as a narrower corolla width could push anthers and the stigma closer together (Dart et al., 2012).

5.2 Materials and Methods

5.2.1 Study system, field sites and common garden plants

Nicotiana glauca (Solanaceae) is a perennial shrub or small tree native to South America (northern Argentina and southern Bolivia) that has become widely naturalised and invasive around the world (Goodspeed, 1954, cited by Nattero & Cocucci, 2007). It has a wide distribution resulting from this species' tendency to colonise tropical and sub-tropical disturbed environments. *N. glauca* grows best under semi-arid conditions and exhibits partial drought and salinity tolerance (Florentine et al., 2006). The long, tubular flowers are somewhat protogynous, with anthers dehiscing 1-2 hours following stigmas becoming receptive (Schueller, 2004) Although flowers are self-compatible (Schueller, 2004) they rely primarily on outcrossing for reproduction in the native range (Ollerton et al., 2012). In native populations the flowers are predominantly pollinated by assemblages of hummingbirds (Nattero & Cocucci, 2007), only occasionally by hummingbird hawkmoths (*Macroglossum stellatarum*) (Issaly et al., 2020) and are visited by non-pollinating *Xylocopa* carpenter bees (Ollerton et al., 2012). In non-native populations in the Americas and Africa, flowers are visited by hummingbirds and sunbirds (Geerts & Pauw, 2009; Ollerton et al., 2012), with bees and flies also making occasional legitimate visits in some populations. Hoverflies, honeybees, native bees and species from the New World bird genus *Diglossa* can also rob the plant for nectar (Ollerton et al., 2012), which the plant produces in considerable amounts of an average of over 5ul per flower in plants in Gran Canaria (pers. comms., Stout 2022). *N. glauca* is not visited by legitimate pollinators in many non-native populations in Europe (e.g., Majorca, Crete and mainland Greece) and the Canary Islands, leading to reliance on self-pollination to achieve reproduction in those populations (Issaly et al., 2020; Ollerton et al., 2012).

We investigated the relationship between floral traits and the capacity for seed set by selfing in plants from both field populations and potted plants grown in a common garden environment. For plants from the field population, we compared the average reproductive output for plants with the measures of corolla traits for flowers, whilst for the potted plants we could relate the SA distance of a single flower to the number of seeds produced by the same fruit. In the potted plants we also compared corolla length, width and flare size with an average reproductive output for plants.

In the field, we studied invasive non-native populations of *N. glauca* in the south-west of the island of Tenerife, Spain, that reportedly have no known pollinators or floral visitors (Ollerton et al., 2012). We focused on two densely aggregated populations of *N. glauca* near Hospital Del

Sur ("Chayofa"; 28.068092, -16.705116) and Chimiche ("El Rio"; 28.137770, -16.530033). Daytime censuses found that no pollinators visited the plant (Ollerton, unpublished data). To record potential evening floral visitors, we also conducted crepuscular census surveys where an observer watched flowers on plants in the Chayofa population between the late afternoon and early evening on three nights in April 2018, and recorded any visits to flowers.

In addition to the field study, we grew plants from different populations in a common garden at the University of Sussex campus, in Brighton, UK. Seed was sourced from plants in the Chayofa population in Tenerife, as well as from a native population in Tupiza (Argentina) and a non-native population in Buffel's River (South Africa). From each population, up to 15 mothers were each used for seed for 10 offspring, with the half-siblings from each mother making up a "family". In April 2019 seeds were sown in 10cm (0.36 L) pots filled with multipurpose compost with sand and reared inside a greenhouse. Plants were arranged in a block design, and replicates from each family and population were split between blocks and randomly assigned a position in a tray within the block. Later in the summer of 2019, the young plants were transplanted to 21cm (4 L) pots. We moved plants outdoors for the summer, back inside the greenhouse to avoid the coldest temperatures during the winter, and then moved outside once again for flowering in summer 2020.

5.2.2 Breeding system and pollen limitation

In the Tenerife field populations we used a hand pollination experiment to determine the breeding system and to test for pollen limitation. Four different treatments were applied to flowers in 17 plants in total, across both the El Rio and Chayofa populations. The treatments were: 1) manual outcrossed (pollen donated from one individual at least 10 metres away); 2) manual selfed (pollen donated by a flower on same inflorescence); 3) autonomous self-pollination (spontaneous selfing); and 4) an open-pollinated (an unmanipulated control). To prevent autonomous self-pollination in treatments 1 and 2, flowers were emasculated by removing anthers with forceps before the flower had opened and anthers dehisced. To be confident that no flower was visited or disturbed by an animal, we covered inflorescences in treatments 1-3 with bridal veil bags.

Approximately 4-6 weeks after treatment, we collected fruits whilst ripe but undehisced, then allowed to dry and dehisce in paper envelopes. We counted the number of seeds produced from each fruit using photographs and a macro designed to count seeds in 1.52e ImageJ software (http://rsb.info.nih.gov/ij/). We compared seed production across treatments using linear

models and pairwise post-hoc Tukey tests in the *stats* package in R (Lenth et al., 2018; R Core Team, 2020).

5.2.3 Floral trait measurements

We measured floral morphology of 6-15 flowers on each of 43 individuals total for the field populations and for six flowers for each of the individuals grown in the common garden sourced from the Chayofa, Tupiza and Buffel's River populations. For flowers in the post-anthesis stage of development we measured floral traits likely to be influencing the propensity of the flower to self, namely the stigma-anther (SA) distance (after anthers dehisce), corolla width, corolla length, corolla flare length and depth (to check for an association between measurements of the flare and SA distance) (Fig 5.1.). If any traits were found to be significantly correlated to each other with a high correlation coefficient ($r \geq 0.4$ within a population), they were analysed as a combined variable calculated as the geometric mean of the two original measures. We measured the SA distance (Fig. 5.1c) as the dimension between the furthest tip of the stigma and the tip of the last anther. Using a DSLR camera we took photographs of flowers set on a stage with 5mm increment graph paper to set a scale. Photos were taken directly above the stage, and flowers were arranged in different positions to allow all measurements from two angles to be taken (Fig. 5.1). Floral traits were then measured in ImageJ software using the straight-line tool.

We measured SA distance of flowers for potted plants that we also collected seed data for, with the aim of relating the SA distance of a single flower to the number of seeds later produced by the fruit from that flower. To do this we labelled flowers with undehisced anthers that were beginning to open, then cut a roughly triangular hole in the corolla to expose the stigma and anthers. Before measuring, inflorescences were bagged for 24 hours, with an uncut flower on the same plant labelled as a control in order to test whether cutting an opening into the corolla affects seed production. SA distance was measured from photos with a DSLR camera, with a scale bar on a ruler attached so the scale could be set in imaging software later. Pictures were taken after all anthers had dehisced and the distance between the longest anther and the end tip of the stigma (Fig. 5.1c) was measured using the straight-line tool in ImageJ. To rule out potential effects of cutting the corolla we compared seed production between treated flowers and control flowers with linear mixed effects models ('lmer', with plant individual as a random factor) using the *lme4* and *lmerTest* packages in R (Bates et al., 2015).
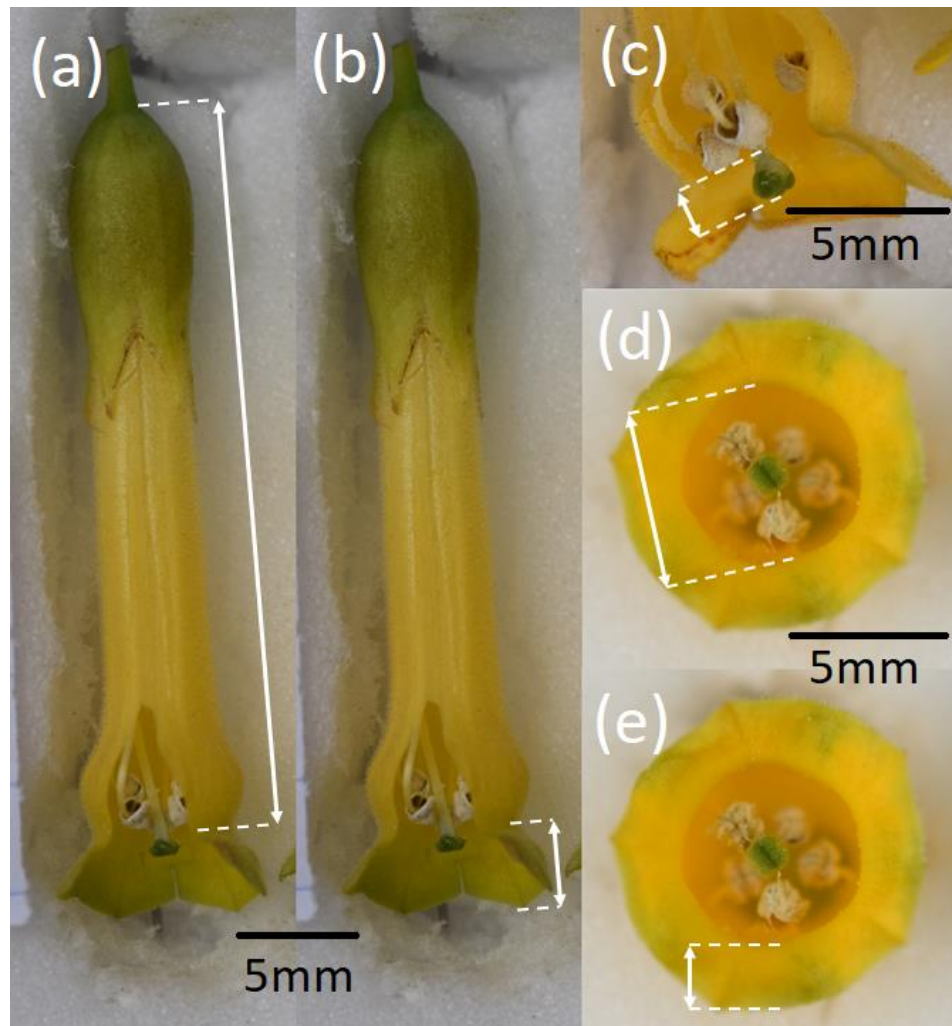
Figure 5.1. Morphology measurements taken for each flower. (a) Corolla length; (b) corolla flare length; (c) stigma-anther (SA) distance; (d) corolla width; (e) corolla flare depth. A portion of the corolla was removed in (a), (b) and (c) for measurement of the SA distance.

To investigate how variable the floral traits were, we used variance partitioning to assess the amount of variation existing between populations, between plants within a population and between flowers within a single individual plant. For this we found the variance component estimates from a linear model ('lme') using the *ape* package in R (Paradis & Schliep, 2019). For the Chayofa population only we compared the variation in floral traits between plants in the field (parents) and their offspring grown under common garden conditions in the greenhouse, using linear models and a Levene's test for equality of variances in R (using the *car* package). One offspring per parent was chosen at random to be included in the comparison, so that similar sample sizes of flowers and plants were compared between groups.

5.2.4 Measuring seed set

Average seed set per fruit for an individual was used as a proxy for selfing success, as selfing is the only means of pollination in the study plants. We measured seed production of fruits for plants in Tenerife and plants in the common garden, for individuals where we have floral morphology data. Between 4 and 12 flowers on each tree were enclosed with bags to prevent interference by any floral visitors (such as nectar-robbing passerines). Ripe but undehisced fruits were collected from each tree around 4-6 weeks after flowers dropped off. Once seeds were collected, they were spread on filter paper and we used a DSLR camera to take photographs. Seeds were then counted from photos using a macro in ImageJ, and seed production per fruit was averaged for each individual tree.

5.2.5. Relationship between floral traits and selfing

We tested for a relationship between corolla traits and seed production at the plant level for 22 plants in Chayofa, 21 plants in El Rio (both in Tenerife) and for 10 plants in the common garden that originated from Tupiza and Buffel's River. For this comparison we used linear mixed-effects models to regress average seed production per tree against floral trait values of individual flowers (with plant individual nested in population as random factors, and plant individual as a random factor in the potted plants), using the *nlme* package in R (Pinheiro et al., 2017).

In addition to testing at the plant level, for 53 flowers across 24 potted plants originating from Tupiza, Chayofa and Buffel's River we were able to directly relate SA distance and seeds produced per fruit by the same flower. For this test of a relationship at the flower level, we used a linear model to test for a relationship between mean seeds and SA distance. However, a high rate of abortion of fruits resulted in an unbalanced design, so we instead used an "lm" instead of a mixed-effects model for this analysis, whilst checking that inclusion of data points from plants sourced from other populations (Buffel's River and Chayofa) did not change the result. In addition to testing for a linear relationship between SA distance and selfing, we also tested for a non-linear relationship (a negative exponential or logistic relationship) using mixed models with the floral variable log-transformed. The fit of these exponential models to the data was compared to that of the linear models by comparing their AIC values and their R squared values (coefficient of determination). To allow a comparison of AIC values, the AIC value for the non-linear model was transformed to be on the same scale as the AIC for the linear model, by

subtracting the sum the logarithms of the response variable (seed production) from the extracted likelihood (Bozdogan, 1987). We found marginal R squared values (R^2) using the 'r.squaredGLMM' function from the package *MuMIn* in R (Nakagawa & Schielzeth, 2013).

5.3 Results

5.3.1 Absence of pollinators

Crepuscular surveys indicated a lack of pollinator visitation to flowers of *Nicotiana glauca*, and surveys of populations in multiple years indicated no legitimate pollination visitation (Ollerton et al., 2012; Ollerton, unpublished). We found evidence of nectar robbing holes made on the flower, possibly by passerine birds we observed landing on the plant. We do not expect that this antagonistic visitation facilitates outcrossed pollination, but it could potentially help aid self-pollination (Rojas-Nossa et al., 2021), along with other biotic and abiotic factors.

5.3.2 Breeding system and pollen limitation in field populations

Hand pollination produced significantly different numbers of seeds across treatments (overall P = 0.027; Fig. 5.2), but the open control (mean = 752 ± 340 SD; N = 14) was not significantly different to any of the treatments in post-hoc comparisons, including manually selfed (mean = 936 ± 214; P = 0.283; N = 11), autonomous self-pollination (mean = 667 ± 210; P = 0.798; N = 16) and manually outcrossed (mean = 883 ± 231; P = 0.484; N = 17). The manually selfed treatment produced significantly more seeds than the autonomous self-pollination treatment (P = 0.043). Additionally, there was no significant difference in seed production between the manually outcrossed and either of the manually selfed (P = 0.949) or autonomous self-pollination treatments (P = 0.080). These results suggest that there is no advantage of outcrossing over self-pollination, in terms of seed production, in these populations. We also demonstrated that *N. glauca* plants in these populations are not pollen limited, as open flowers can produce as many seeds as hand-pollinated ones. We found that the control fruits were more variable (variance = 115426; N = 17) in the number of seeds they produced than for all other treatments (variances were between 44109 and 53121 for the three hand pollination treatments).
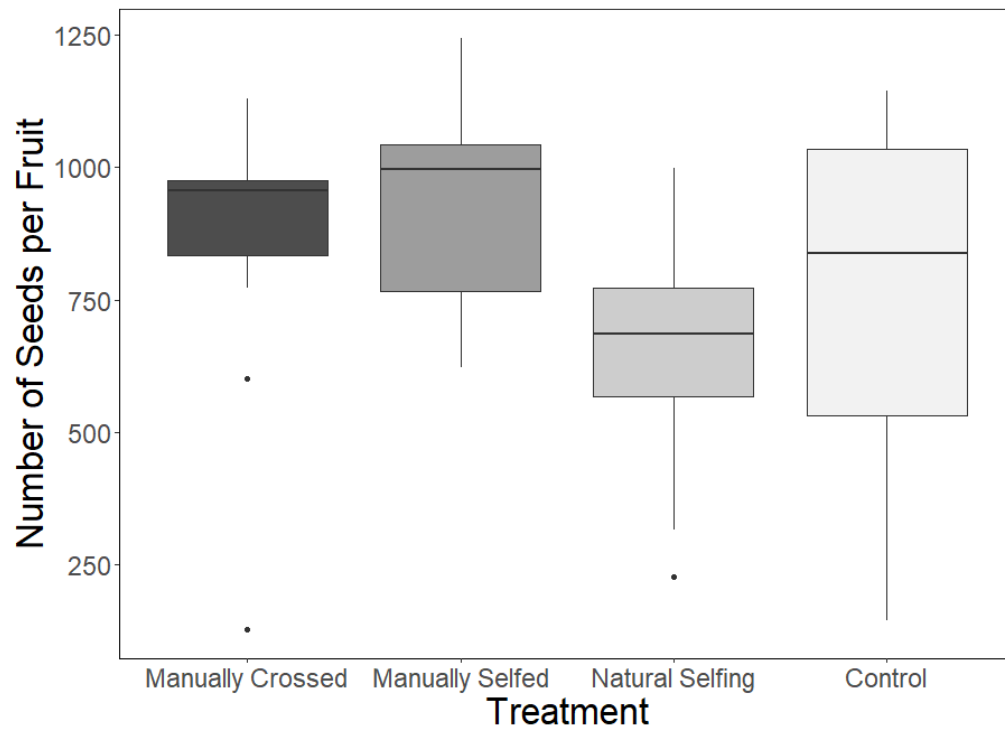
Figure 5.2. Seed production after four hand pollination treatments (N = 11-17 plants) administered to *Nicotiana glauca* flowers in the Tenerife field populations.
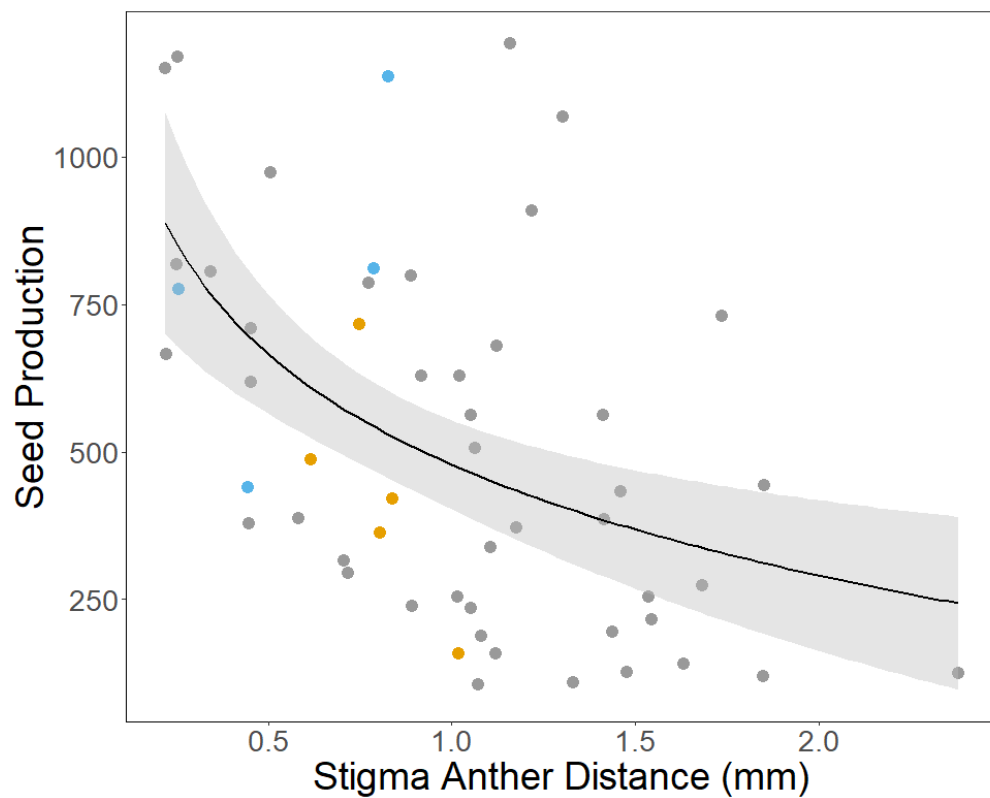
Figure 5.3. Non-linear relationship between stigma anther (SA) distance and seed production in the same flowers, from plants grown in a common garden. Plants were sourced from mothers in Tupiza in Argentina (grey), Chayofa in Tenerife (orange) and Buffel's River in South Africa (blue).
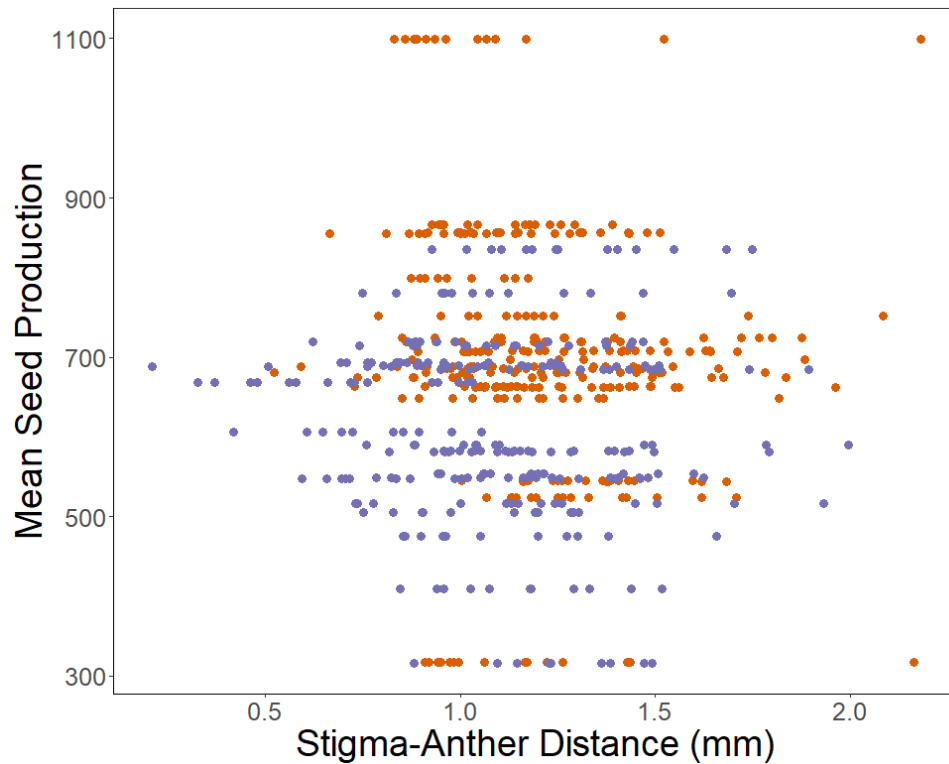
Figure 5.4. Relationship between the mean number of seeds per fruit and stigma anther (SA) distance of flowers across two populations in Tenerife, Chayofa (orange) and El Rio (purple).

5.3.3 Floral trait correlations and variability

Across individuals in the field populations, we found that SA distance was significantly correlated with corolla width (Pearson r = -0.18, P < 0.001, N = 532) but not with any other floral trait. There were strong correlations between the corolla tube flare length and depth (r = 0.27 across populations and r = 0.51 in El Rio, both P < 0.001; N = 536) so these two measures were combined into a geometric mean termed "corolla flare size". In the absence of strong correlations between all other traits measured (no correlation coefficient exceeded 0.35) we analysed corolla length, corolla width and SA distance separately to infer their individual role in pollination success.

Variance partitioning analysis on plants in the field populations found that most of the variation in SA distance was across flowers within a plant (76.7%; Table 5.1; Appendix Fig. A5.1) whilst there was lower variability between plants (17.2%) or between populations (6.2%). The majority of the variation in corolla flare size was again within plants (61.3%) whilst most of the variation in corolla length was between plants (54.2%) with a sizeable amount of variation being between flowers at the level of the plant (37.6%). In contrast, the greatest amount of variation in corolla width was between populations (61.1%), with very little variation for this trait being within

(19.6%) or between plants (19.3%; Appendix Fig. A5.1). Similar trends were found for variance partitioning in potted plants for SA distance and flare size (Table 5.1). For corolla length in potted plants, most of the variation was between flowers within plants (94.4%) rather than between plants (5.6%). For corolla width the variation was comparable within (50.8%) as between plants (49.2%).

We used a Levene's test to check for equal variances in floral trait measures for field plants from Chayofa and potted plants grown from Chayofa parents. This found that the variance in corolla length in the field (σ^2 = 2.375; N = 84; P < 0.001) was higher than that for potted plants (σ^2 = 1.101; N = 90). Corolla width was not more variable in the field (σ^2 = 0.295; N = 84; P = 0.322) than in the potted plants (σ^2 = 0.264; N = 90). The variance in SA distance in the field (σ^2 = 0.090; N = 84) was not found to be greater than SA distance in the potted plants (P = 0.178; σ^2 = 0.155; N = 83; Appendix Fig. A5.2) although both were very small. The variance in corolla flare size was comparable in the field (σ^2 = 0.137; N = 78) as in the potted plants (P = 0.229; σ^2 = 0.130; N = 90)

5.3.4 Relationship between floral traits and selfing

In common garden plants, we found a significant non-linear negative relationship between SA distance and mean seeds produced by the same flower (P < 0.001; N = 53; Fig. 5.3), and the relationship holds when removing one data point at the bottom of the curve (Fig. 5.3). The non-linear model was a better fit for the data compared to a linear model as the non-linear model has a lower AIC value (AIC = 739.16, R^2 = 28.3%) than the linear model (AIC = 750.56, R^2 = 23.2%).

Our test in potted plants found that seed production (i.e. the rate of selfing) and floral traits measures did not show a relationship at the level of the plant, for corolla length (P = 0.059; N = 56; Appendix Fig. A5.3), corolla width (P = 0.135; N = 46; Appendix Fig. A5.3) or corolla flare size (P = 0.5617; N = 46; Appendix Fig. A5.3). Our manipulation of cutting a hole into the corolla tube did not affect the seed production, as manipulated flowers (N = 44) did not produce significantly different numbers of seeds to the untouched flowers on the same individual's control treatment (N = 11, P = 0.743).

When testing for a relationship between SA distance and the average number of seeds produced by selfing at the individual level in field populations in Tenerife, we did not find a significant relationship (P = 0.051; Fig. 5.4). Similarly, the analysis did not find a significant relationship between selfing and corolla length (P = 0.175; N = 579), corolla width (P = 0.142; N = 571), or corolla flare size (P = 0.091; N = 570; Appendix Fig. A5.4).

Table 5.1. Variance partitioning (%) of the floral traits investigated in this study

	Level of Variation	SA distance	Corolla Length	Corolla Width	Flare Size
	Within Plants	76.7	37.6	19.6	61.3
Field Populations	Between Plants	17.2	54.2	19.3	37.4
	Between Populations	6.2	8.2	61.1	1.4
Common garden (Chayofa population)	Within Plants	78.7	94.4	50.8	64.9
	Between Plants	21.3	5.6	49.2	35.1

5.4 Discussion

Our study investigated the relationship between floral traits and selfing in *Nicotiana glauca* at the individual plant and flower level, to understand the mechanical aspects of selfing and the role of phenotypically plastic variation in the outcome of this relationship. We found evidence of a significant negative and non-linear relationship between SA distance and the propensity of flowers to self-pollinate at the level of the flower in the common garden. However, this was not the case when looking at the same relationship at the level of individual plants in field populations. No significant relationship was found for other floral traits thought to be important in influencing selfing, despite corolla tube width being expected to play a role. SA distance showed a high degree of variability with 76.7% of the variation being within plants. We also found that corolla flare size had a high plastic variation within individuals, whilst corolla length and width had most variation between population which could indicate stronger genetic control. Below we discuss the implications of high within- individual plasticity in SA distance that leads to the relationship between SA distance and selfing not being detectable or present at the level of the plant.

Our finding of a significant non-linear relationship between SA distance and selfing for single flowers in the common garden is consistent with a review by Opedal (2018) that found many autofertile species and individuals with reduced herkogamy (including SA distances) have increased rates of selfing. We assume the underlying mechanism is that with increasing proximity of stigma and anthers, the probability of contact between them increases and hence flowers have a higher rate of self-pollination (Kalisz et al., 1999). In studies on other plant

species, flowers with a shorter SA distance have been shown to have more pollen grains deposited on stigmas (Jiang et al., 2018) and also produce a greater number of seeds (Eckert & Schaefer, 1998). In *N. glauca* we find that the relationship between herkogamy and selfing is an exponential decay where the number of seeds being produced steeply declines at low SA distances, whilst there are at least some seeds produced at high SA distances. We have demonstrated that pollen may not be a limiting factor for the flowers, with at least some pollen from anthers contacting conspecific stigmas regardless of the SA distance. The pollen may be travelling within a flower or the anthers are contacting the stigma at some point, such as could happen when a flower wilts during senescence (Dole, 1990). Although we find a relationship between SA distances and seed production from selfing in individual flowers, we did not find such a relationship when testing at the level of the plant.

The lack of a relationship between SA distance and selfing at the level of the plant individual in field populations could be explained in part by the large amount of plastic variation found within individuals for this trait. Although high plasticity could be unexpected for floral traits related to pollination success (precise pollen transfer is required for fertilisation in many specialist species; Armbruster et al., 1999), we found a large amount of variation in SA distance within plants (76.7% of all variation in the population), which could be a response to abiotic factors such as temperature (Razanajatovo et al., 2020) and soil moisture content (Mal & Lovett-Doust, 2005). Therefore, since the individual is the unit that selection acts upon then there is unlikely to be a strong response to selection for decreased SA distances to achieve greater seed production (Opedal et al., 2017). The high plasticity in traits relating to breeding may be contributing to the success of *N. glauca* (Leibman et al., 2018; Pannell, 2015)*,* as the species is able to persist in dense aggregations in populations around the globe that lack pollinators. However, this runs counter to the pattern found by Ollerton et al., (2012) that mean SA reduced predictably from native populations to non-native bird-pollinated populations to non-native selfing populations in the Canary Islands and southern Europe, a pattern interpreted by those authors as being driven by natural selection for selfing. However, the range of SA distances recorded in our study (0 - 2.5mm) do not represent the entire variation of the trait across the globe that can be up to 3.5mm in native populations (Ollerton et al., 2012).

Our test on field population plants found a high level of residual variance in mean seed production not explained by SA distance, which could be explained by one or a combination of unknown abiotic factors such as wind, humidity, temperature and precipitation (Vogler et al., 2010). In our hand-pollination experiment, flowers in the bagged treatments 'manually outcrossed' and 'manually selfed' set 31.4% and 40.8% more seeds (respectively) than 'naturally

selfed' flowers that were bagged but not hand pollinated (presumably, to autonomously self). Therefore, in inflorescences covered with bags, not enough pollen grains are reaching stigmas within a flower for it to achieve full seed set. Additionally, we found that unbagged and unmanipulated flowers (the control group) set a much more variable number of selfed seed than treatments involving bagging. The action of wind and/or other biotic or abiotic factors (such as disturbance from nectar robbers) could potentially be helping to dislodge and move around pollen (Qu et al., 2007; Wang et al., 2004). Future work could help assess the effect of wind (and other factors) on influencing self-pollination in this plant.

For other floral traits measured in this study, including corolla length and width, we found no significant relationships with selfing. We expected to find a link between selfing and a linear measure of flower size such as corolla length, since Dart et al., (2012) found that transitions in the mating system of *Camissoniopsis cheiranthifolia* towards selfing involved a sizeable reduction in flower size alongside reductions in herkogamy. Similarly, Brys et al., (2013) and Carleilal et al., (2017) found that, selfing populations of *Blackstonia perfoliata* and *Arabidopsis lyrata* had 28.5% and 9.2% smaller flowers, respectively, when compared to non-selfing populations. These reductions in flower size alongside selfing can be expected, as the switch to selfing can often cause directional selection for larger corolla traits to become relaxed (or selection for smaller corollas because they cost less to produce) due to the resource cost (Galen, 1999; Teixido & Valladares, 2013; Vallejo-Marín & Barrett, 2009). If SA distance is the single and/or most important floral trait that directly influences selfing, then it stands to reason that other corolla traits (length, width and flare size) are not likely to play a major role in the rate of selfing. However, further work could assess whether there is a direct relationship between these corolla traits and selfing at the level of the flower, and if, like SA distance, there is no relationship found at the level of the plant individual.

5.4.1. Concluding Remarks

This study finds that SA distance is a factor influencing the propensity of *N. glauca* flowers to self, which is consistent with studies on other species (Brys & Jacquemyn, 2011; Opedal, 2018). This contributes to a mechanistic explanation of how plants in pollinator-absent non-native populations of *N. glauca* have predominantly switched to selfing (Ollerton *et al*., 2012), with other factors such as wind (Qu et al., 2007; Wang et al., 2004) and curvature of the corolla also potentially playing a role (García et al., 2020; Issaly et al., 2020). Although we could expect selection on traits that influence selfing as this is the only means of reproduction, the high

amount of plastic variation with plants decreases the likelihood that there can be a rapid response to selective pressures for decreased SA distances when pollinators are absent. If selfing is unlikely to rapidly evolve in non-native populations as the predominate reproductive strategy, then it is important that plasticity of this trait remains high so that at least some flowers in plants are producing seed by selfing. Further enquiry into how plant reproduction and floral morphology respond to the loss of animal pollination will be important in the face of accelerating pollinator decline.

Chapter 6

Plasticity and heritability of *Nicotiana glauca* floral traits in a common garden

Abstract

When animal pollination becomes unreliable, such as when plants colonise new areas, self-compatible species rely on self-pollination to ensure reproduction. From prior studies we expect changes in herkogamy (the spatial separation between stigmas and anthers within a flower), and other corolla traits to be influencing the likelihood of the transition from outcrossing to predominantly self-pollination. However, the capacity for floral traits to respond to new selective pressures depends on their genetic basis, but we do not properly understand how the variation in floral traits is determined at the population level, and whether phenotypic plasticity plays a role in this variation. In this chapter we studied the floral trait variation of *Nicotiana glauca* plants in a common garden experiment, grown from seeds sourced from different native and non-native populations around the globe. We compared corolla length, corolla width and stigma-anther distance in the common garden plants with plants from field populations from which they originate. We calculated the relative distance plasticity index (RDPI) as a measure of the phenotypic plasticity of floral traits. Additionally, we used parent-offspring regression to calculate the narrow-sense heritability of traits for two populations in the common garden, with a correction to account for inbreeding effects for selfing. We found significant heritabilities for all floral traits in plants from one population of origin, ranging from 0.16 to 0.29, but not for any traits for plants originating from the other population. Stigma-anther distance showed high levels of phenotypic plasticity, indicated by high RDPI values and evidenced by the variation for populations tightly clustering around a global mean for the common garden plants as opposed to the field population plants. This study confirms that stigma-anther distance varies plastically in *N. glauca* and may help explain the reproductive success following invasion.

Key Words: self-pollination, herkogamy, plasticity, heritability, common garden

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6.1 Introduction

Following changes in the visiting pollinator assemblage, plants can ensure continued pollination through rapid adaptation (Mackin et al., 2021; see Chapter 1) and plasticity of the mating system (Leibman et al., 2018; Peterson & Kay, 2015). One such situation is in invasive or colonising plants, where animal pollinators that plants once relied on for outcrossing can become unreliable in the new range. In contrast to the naturalisation of *Digitalis purpurea* in the Americas, where an additional and novel pollinator functional group is gained (see Chapter 2), a complete loss of pollination services can lead to reliance on self-pollination as a means of achieving reproductive success (Pannell, 2015). Selfing species often show floral traits that increase the chances of successful self-fertilisation, such as reduced herkogamy (the distance between anthers and stigmas in hermaphroditic flowers) compared with outcrossing species (Opedal, 2018). *Nicotiana glauca* (the tree tobacco), for instance, recently transitioned to selfing in some populations following pollinator loss (Ollerton et al., 2012). In Chapter 5, we showed that there is a direct mechanical relationship between the stigma-anther distance (SA distance) and the rate of selfing in individual flowers of this species. However, selfing is not explained by the mean SA distance of individual trees growing in the wild, which show a high intra-individual variation in this and other floral traits. This suggests that the traits are not under strong genetic control.

For traits that are closely related to reproductive success, theory predicts that their variation should be under genetic control (Alonso-Blanco et al., 1999; Mitchell & Shaw, 1993) and as a consequence, floral traits are often expected to have high heritabilities. In many previous studies, this is indeed the case, such as for corolla length, corolla width, stigma position and anther position (Campbell, 1996; recently reviewed by Opedal, 2019). However, for herkogamy in selfing populations, heritability (and also evolvability; see Chapter 3) can be very variable among species, ranging from 0.02 and 0.84 (Opedal et al., 2017; N = 16 studies; see Appendix Table A6.1). High intra- and inter-individual variation could be favoured if, in plants that rely on both outcrossing and selfing, flexibility of the mating system is achieved through plasticity of floral traits and provides reproductive assurance (Peterson & Kay, 2015). This could be the case

in invasive plants for example, if inter-individual variation in floral traits favours invasiveness by providing plants the flexibility of selfing in the absence of pollinators. Here we explore the sources of variation in floral traits that influence selfing in invasive *N. glauca*, to test this possibility.

Nicotiana glauca has successfully invaded areas of the world where no pollinators are available for the bird-specialised flowers. In areas such as Mallorca, the transition from predominantly outcrossing to predominantly selfing in pollinator-poor areas could be an evolutionary adaptation, since other invasive areas visited by pollinators do not have such a high capacity for selfing (Issaly et al., 2020). The study by Issaly et al., (2020) did not find a clear role of herkogamy in explaining SA distance when testing for an association between these two variables at the level of the individual. However, our previous work testing for a relationship at the level of single flowers shows that shorter stigma-anther distances are influencing the higher rates of selfing (Chapter 5), presumably due to the increased probability of pollen contacting conspecific stigmas when it is released from anthers at a shorter distance from stigmas (Jiang et al., 2018; Kalisz et al., 1999). However, there is high variability in SA distance across flowers of the same plant, and this plastic variation could be explaining why populations across the world have not shown adaptive evolution of herkogamy and other floral traits.

The aim of this study was to examine to what degree the floral trait variation in *N. glauca* can be explained by additive genetic variance and thus to what extent the variation is influenced by environmental conditions (through plasticity). We utilised a common garden to grow plants of *N. glauca* sourced from populations with and without animal pollination in common environmental conditions. All plants had known maternal phenotypes. We assessed the degree of phenotypic plasticity, and estimated the heritabilities of traits in the common garden using a parent-offspring regression, similar to methods in Chapter 3. We focus on herkogamy (SA distance) but also on corolla traits for comparison.

6.2 Methods

6.2.1 Study system

Nicotiana glauca (Solanaceae) is a perennial shrub native to South America (southern Bolivia and northern Argentina) and naturalised in over four continents (Goodspeed, 1954, cited by Nattero & Cocucci, 2007). The plant typically grows well in arid conditions, with partial tolerance to drought and saline conditions (Florentine et al., 2006) and areas the plant has established in

the non-native range include Spain, Greece and the Canary Islands (Ollerton et al., 2012). Although *N. glauca* relies primarily on hummingbird pollination for outcrossing in the native range (Garcia et al., 2020) the plant is self-compatible and relies on self-pollination in many non-native populations that lack pollinators (Issaly et al., 2020; Schueller, 2004). The long, tubular flowers require animals with long mouthparts to reach the nectaries to feed from and pollinate it, resulting in many invasive populations without an appropriately long-tongued pollinator.

6.2.2 Common garden setup

To compare how the floral trait variation is expressed in a common environmental setting with that of the parental field populations, we grew plants sourced from different populations in a common garden at the University of Sussex campus. Use of a common garden allows reduction of environmental differences and insight into how plants are genetically different from one another. Included in comparisons are plants sourced from native populations visited by hummingbirds (Tupiza in Argentina and Piedra Pintada in Bolivia), a non-native population visited by sunbirds (Buffel's River in South Africa) and non-native populations not visited by pollinators (Génova in Mallorca and Chayofa in the Tenerife). Further details of the populations and locations sampled from can be found in Table 6.1.

We grew 10 offspring each from 15 parents from each of the five populations, with the offspring from each parent hereon referred to as a "family". Plants were sown during April 2019 and initially reared inside greenhouses at the University of Sussex. Plants were initially grown in 15cm (0.5L) pots filled with multipurpose compost and sand. Pots were arranged in a block design, with plants from families allocated to each block, with randomly assigned position within trays in blocks. Later in summer 2019, plants were transplanted to 21cm (4L) pots. The pots were moved outdoors for the summer, moved back inside greenhouses during the winter to avoid freezing temperatures and moved outside once again in Summer 2020 for flowering.

6.2.3 Trait measurements

We measured floral traits in the parental field populations (Table 6.1) and common garden plants, including those investigated in previous research: the corolla length, corolla width and SA distance (see Chapter 5). Floral traits were measured in replicate flowers from each plant – up to five flowers per plant in Tupiza and Piedra Pintada, between three and five flowers in Buffel's River and Génova and up to six flowers in Chayofa. Measurements of the parental field

population in Génova are not available, so this population of origin was not included in inter-generational comparisons.

For Buffel's River, Tupiza and Piedra Pintada, corolla length and corolla width were found by measuring the distance between landmarks in MorphoJ (see Fig.1 in Issaly et al., 2020). For Chayofa plants and all common garden plants, distance between the same landmarks was measured (see Fig. 5.1 in Chapter 5) using a straight-line tool in ImageJ (http://rsb.info.nih.gov/ij/). Photos were taken with a DSLR camera on a stage with a scale bar (1mm increment graph paper).

For SA distance measurements, flowers were photographed from the side with a DSLR camera following excision of the corolla to expose the reproductive parts (see Fig 5.1 in Chapter 5). SA distance was measured as the distance between the tip of the stigma to the tip of the longest anther. For Buffel's River, Tupiza and Piedra Pintada measurements were made with SigmaScan Pro 5.0 software (SPSS Inc.). For Chayofa and all plants in the common garden, a similar method was used in ImageJ to measure SA distance, using the straight-line tool.

6.2.4 Trait variation in the common garden

We used a variance partitioning analysis for each population in turn to find how much of variation in floral traits was found among families, among plants and among flowers within plants. For this we used linear models ('lme') using the *nlme* and *ape* packages in R (Paradis & Schliep, 2019).

We ran a mixed-effects linear model ('lmer') to test for differences between trait means for plants sourced from the introduced range and plants sourced from the native range, using the *lme4* package in R (with population as a random factor).

6.2.5 Comparing floral traits between common garden and parental populations

We compared the floral trait variation of each group grown in the common garden with their corresponding population of origin. To do this, we used mixed-effects linear models ('lmer') to compare field and common garden data with the family group as a random factor. We also tested whether the floral trait variation in the common garden clusters more closely together around a common mean (across all populations) as compared with variation in field population plants. A Shapiro-Wilk test first confirmed that the three floral traits do not follow a normal

distribution (P < 0.001). We thus used a non-parametric Levene's test (from the *car* package) to check for equal variances across all field plants and all common garden plants for each trait in turn. If the floral trait values converge around a common mean more so in the common garden than in the field populations, then at least some of the variance in floral traits is a response to differing environmental conditions.

6.2.6 Floral trait plasticity

For the populations with a sufficiently large sample sizes (Buffel's River Chayofa and Tupiza), we estimated the indices of plasticity from the two different growth environments (field and common garden). We computed the relative distance plasticity index (RDPI), a robust metric used in statistical comparisons of plasticity where values range from 0 (no plasticity) to 1 (maximum plasticity). The calculation is of relative distances of trait values between parent and offspring pairs of plants grown in different environments, where the absolute value of the differences in trait values is divided by the sum for all pairs of individuals grown in different environments. The measure is calculate by, RDPI = $\sum(d_{ij}\rightarrow i'j'/(x_{i'j'} + x_{ij}))/n$ (where $d_{ij}\rightarrow_{i'j'}$ is the distance among trait values, $x_{i'j'} + x_{ij}$ is the differences of the sum and n is the total number of distances; Valladares et al., 2006). We balanced the dataset by randomly choosing one offspring per family to be compared with the parental values. For plants from Buffel's River and Tupiza, measures of three flowers per offspring were included, whilst six values per offspring were included for analysis of plants sourced from Chayofa, since more floral measurements were available. The RDPI values were calculated for each population for corolla length, corolla width and SA distance with the *ameztegui/Plasticity* package in R (Ameztegui, 2017). Typically studies calculating the RDPI use individuals with the same genotypes (clones) although the method is also valid for individuals with relatedness such as half siblings (Valladares et al., 2006), or in our case, parents and offspring. We compared RDPI values between the native and introduced range for each trait with a 'lmer' (with population as a random factor), and between the two populations within the introduced range with a linear model. To find if the traits themselves differed in plasticity, we also compared values between traits with a 'lmer', with population as a random factor.

6.2.7 Estimating heritability of floral traits

We used parent offspring regression to estimate the heritability of the corolla length, corolla width and SA distance separately for plants originating from Buffel's River and Chayofa. We used linear regression ('lmer') of the parental values of the trait against the average offspring values (averaged for each individual) with the family group (parent) as a random factor. We found the coefficient of the slope and the confidence interval, and from this we estimated heritability of each trait. We assumed plants in the Buffel's River population are mostly outcrossing, due to high rates of bird pollination and the low fruit set when bird-visited flowers are forced to autonomously self by bagging (Issaly et al., 2020). Therefore, we also assume plants sourced from this population in our common garden are half-siblings. With only values for one of the parents, we multiplied the slope of the regression line by two to find the narrow-sense heritability (Fernandez & Miller, 1985; Smith & Kinman, 1965).

We also estimated the heritability of floral traits for plants sourced from a selfing population, Chayofa. For this study we will assume the inbreeding coefficient (F) of the selfing population (Chayofa) is 1, due to there being no reports or instances of pollination visits during past censuses. As before we used mixed-effects models ('lmer') to calculate the slope of the parent-offspring regression. To account for the overestimation of heritability resulting from inbred offspring, we corrected the slope using the equation b' = b / [1 + F (1 - b)] where b' is the strict narrow sense heritability, b is the regression coefficient and F is the inbreeding coefficient which we assume to be 1 (Gibson, 1996; Holland et al., 2003; Nyquist, 1991; Smith & Kinman, 1965). Although this correction does not account for non-additive GxE effects, the inclusion of this correction for inbreeding will allow for a more accurate estimation of heritability to be made.

6.3 Results

6.3.1 Trait variation in the common garden

In partitioning the variance we determined the amount of variation at the level of the family, individual plant and within plants for corolla length, corolla width and SA distance for each population (Fig. 6.1). For SA distance, we found that most of the variation was within plants and not between plants or families, for Buffel's River (81.3%; N = 641), Chayofa (85.6%; N = 639), Tupiza (70.7%; N = 252) and Génova (89.2%; N = 271; Appendix Table A6.2) but not for Piedra Pintada (26.8%; N = 135).

Comparisons between the introduced and the native parts of the range did not show significant differences for the corolla length (P = 0.053; N = 1945 flowers; Fig. 6.2a) or for the SA distance (P = 0.119; N = 1873; Fig. 6.2c) for plants in the common garden. However, we did find that the corolla width is significantly larger for plants in the common garden sourced from the native range than for plants sourced from introduced populations (estimate = 0.4595; N = 1871; P = 0.002; Fig. 6.2b).

6.3.2 Comparing floral traits between the common garden and parental populations

In most cases, floral traits were larger in the common garden than for plants from the parental field populations. For Buffel's River, floral traits were significantly larger in the common garden than in the parental field populations for corolla length (P < 0.001; Fig. 6.3a), corolla width (P = 0.005; Fig. 6.3b) and SA distance (P < 0.001; Table 6.2; Fig. 6.3c). In Chayofa the common garden plants showed larger values than field plants for corolla length (P < 0.001; Fig. 6.3a) and corolla width (P < 0.001; Fig. 6.3b) but not for SA distance (P = 0.202; Table 6.2; Fig. 6.3c). For Piedra Pintada, only the corolla width was significantly larger in the common garden than the field populations (P = 0.022; Fig. 6.3b), whilst the corolla length (P = 0.667; Fig. 6.3a) and SA distance showed no difference (P = 0.776; Table 6.2; Fig. 6.3c). In Tupiza corolla width (P = 0.047; Fig. 6.3b) and SA distance (P < 0.001; Table 6.2; Fig. 6.3c) were larger in the garden than in the field, but corolla length was smaller in the garden than the field (P < 0.001; Fig. 6.3a).

A Levene's test for equal variances found that for two floral traits out of three, the variation clustered more closely around a common mean for plants grown in the common garden than for field population plants. We found that the corolla length values are distributed closer around a global mean in the common garden (mean = 37.03mm, σ^2 = 11.27, N = 1643; P < 0.001) than for the field population plants (mean = 36.48mm, σ^2 = 19.82, N = 302 flowers). Similarly, the values for corolla width converged more closely around a common mean in the common garden (mean = 6.48mm, σ^2 = 1.21, N = 1569; P = 0.003) than in the field plants (mean = 6.14mm, σ^2 = 1.49, N = 302 flowers). However, there was no significant difference in the variance of SA distances between the common garden (mean = 1.26mm, σ^2 = 0.32, N = 1570; P = 0.491) and the field population plants (mean = 1.16mm, σ^2 = 0.355, N = 303).

Table 6.1. Populations we sourced *Nicotiana glauca* plants from for floral traits data and for seeds for the common garden.

Region	Population	Coordinates (latitude, longitude)	Elevation (m)	Field data	Common garden data	Heritability Estimate
Argentina (native)	Tupiza	-21.6775, -65.578611	3155	Yes	Yes	Yes
Bolivia (native)	Piedra Pintada	-29.429444, -67.829722	1573	Yes	Yes	Yes
South Africa (non-native)	Buffel's River	-29.720278, 17.614722	202	Yes	Yes	No
Mallorca (non-native)	Génova	39.350278, 2.390278	100	No	Yes	No
Tenerife (non-native)	Chayofa	28.068092, -16.705116	189	Yes	Yes	Yes
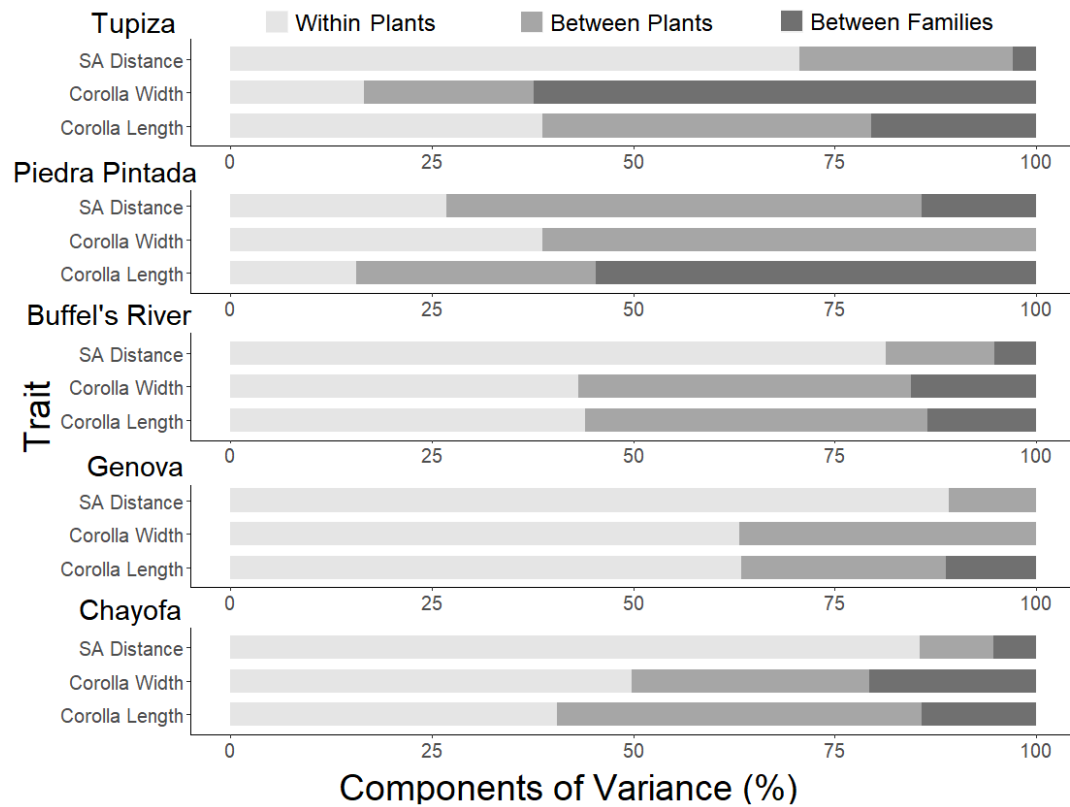
Figure 6.1. Variance Partitioning for the floral traits in this study, for each native (Tupiza and Piedra Pintada) and non-native (Buffel's River, Génova and Chayofa) population.
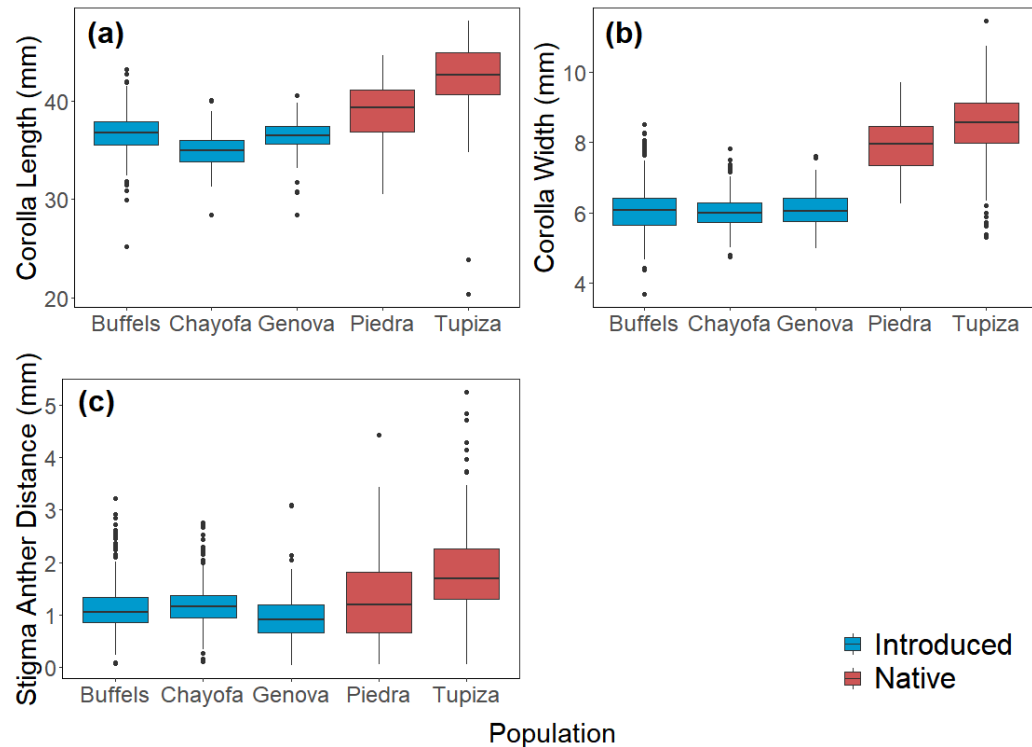
Figure 6.2. Floral traits for the common garden plants sourced from introduced and native populations, including (a) corolla length (N = 1910 flowers); (b) corolla width (N = 1835) and (c) SA distance (N = 1822).

6.3.3 Floral trait plasticity

When comparing RDPI values between ranges, we find that corolla length is more variable in the native range (mean = 0.069 ± 0.072 SD; P = 0.019; N = 8 plant pairs) than in the introduced range (mean = 0.033 ± 0.018; N = 28; Appendix Fig. A6.2). The corolla width in the native range (mean = 0.077 ± 0.054; P = 0.417; N = 8 plant pairs) is not significantly different from the introduced range (mean = 0.063 ± 0.038; N = 28; Appendix Fig. A6.2), and similarly the SA distance does not show significant differences between the native (mean = 0.228 ± 0.176; P = 0.944; N = 26 plant pairs) and introduced range plants (mean = 0.219 ± 0.129; N = 26; Appendix Fig. A6.2).

We found higher values of RDPI for SA distance in plants sourced from Buffel's River (mean = 0.277 ± 0.156 SD; P < 0.001; N = 13 plant pairs) than in Chayofa (mean = 0.162 ± 0.054; N = 13; Appendix Fig. A6.2). However the values of plasticity for corolla length were not significantly different in Buffel's River (mean = 0.036 ± 0.02; P = 0.409; N = 13) as in Chayofa (mean = 0.031 ± 0.016; N = 14; Appendix Fig. A6.2). Similarly, the values of plasticity for corolla width did not differ between Buffel's River (mean = 0.060 ± 0.047; P = 0.607; N = 14) and Chayofa (mean = 0.067 ± 0.029; N = 14; Appendix Fig. A6.2).

When comparing RDPI values between traits, we found that SA distance (mean = 0.221 ± 0.137; N = 33 plant pairs) showed more plastic variance than corolla length (mean = 0.041 ± 0.039; P < 0.001; N = 36) and corolla width (mean = 0.066 ± 0.042; P < 0.001; N = 36). Corolla length and corolla width did not differ significantly in RDPI values (P = 0.295).

6.3.4 Estimating heritability of floral traits

We found significant heritabilities for all three floral traits when regressing offspring against parental values for plants sourced from Chayofa and no significant heritabilities for any traits for plants sourced from Buffel's River. We found significance for heritability for corolla length in Chayofa (h^2 = 0.16; P = 0.037; N = 99 parent offspring comparisons) but not in Buffel's River (h^2 = 0.85; P = 0.055; N = 102; Fig. 6.4). We found significant heritability for corolla width in Chayofa (h^2 = 0.17; P = 0.038; N = 99) but not in Buffel's River (h^2 = 0.27; P = 0.432; N = 102; Fig. 6.4). We also found significant heritability for SA distance in Chayofa (h^2 = 0.29; P = 0.047; N = 98), but not in Buffel's River (h^2 = -0.04; P = 0.907; N = 102; Fig. 6.4; Table 6.3).
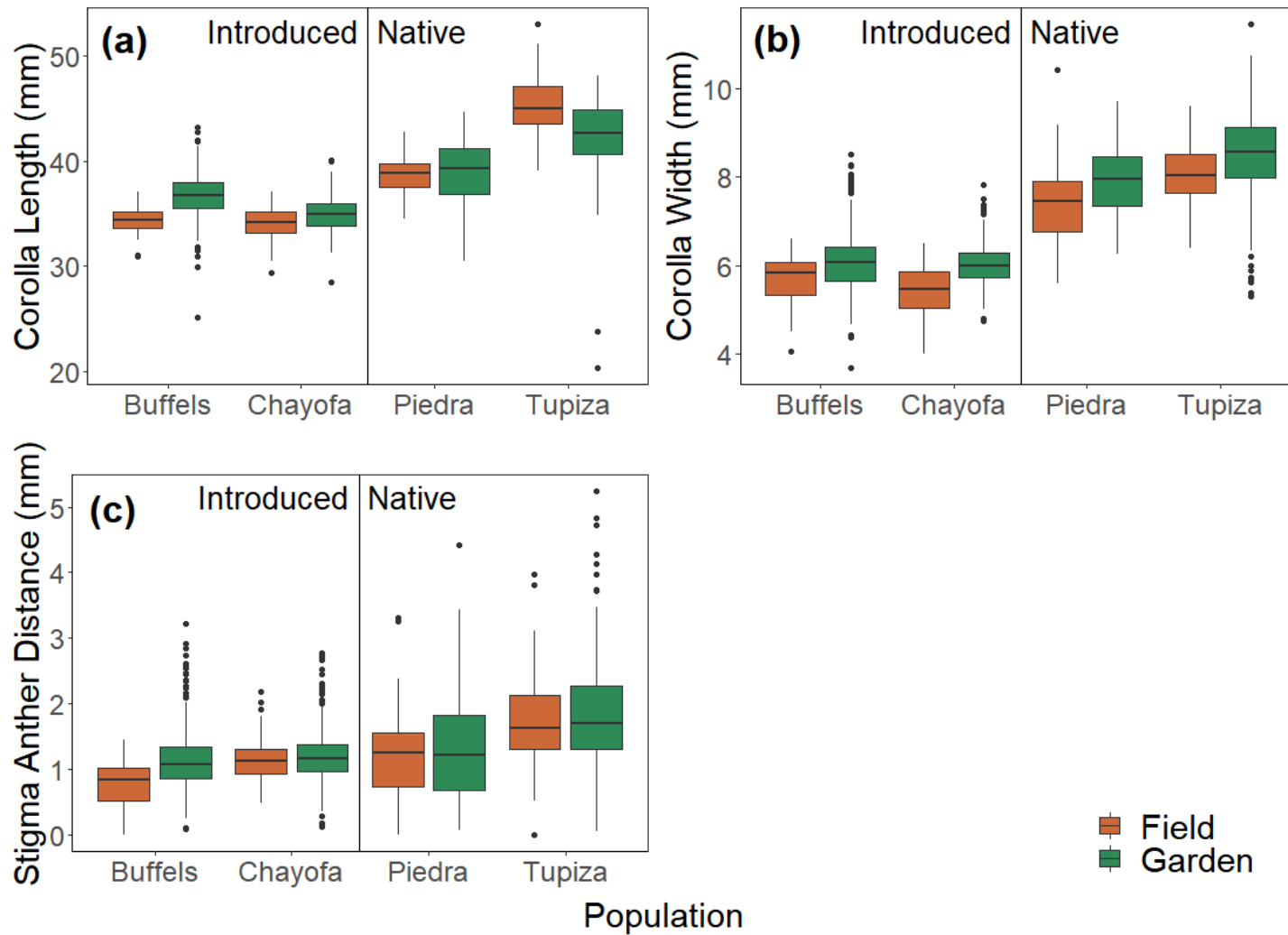
Figure 6.3. Floral trait measurements for (a) corolla length in field populations (N = 302) and common garden (N = 1643) (b) corolla width in field populations (N = 302) and common garden (N = 1569) and (c) SA distance in field populations (N = 303) and common garden (N = 1570).
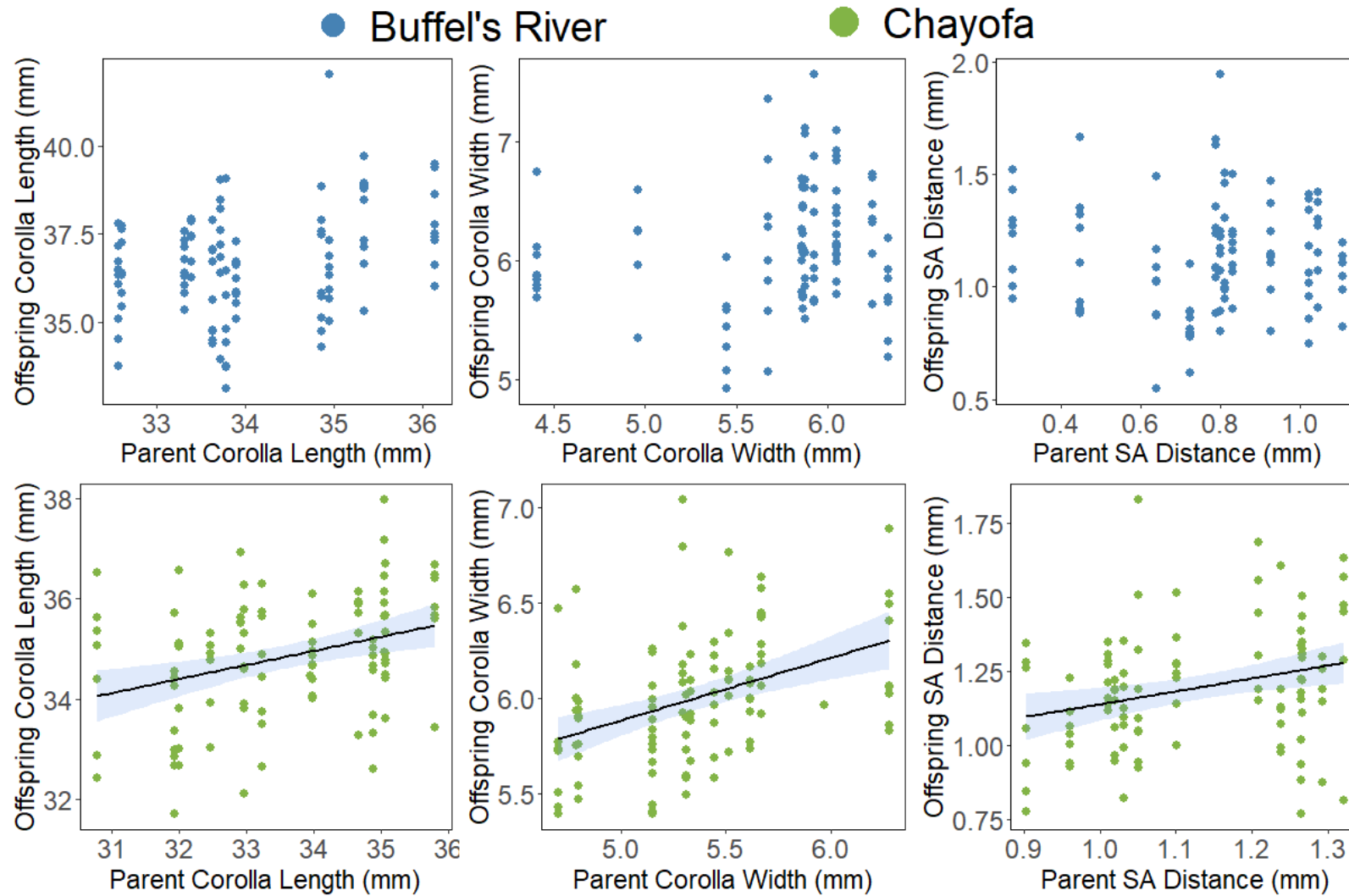
Figure 6.4. Parent-offspring regression of floral traits from field parents and their offspring in the common garden for the traits corolla length, corolla width and SA distance. Buffel's River (top; N = 102 offspring) and Chayofa (bottom; N = 99 offspring).

Table 6.2. Floral trait variation for field population and population in the common garden. Values included are the means, standard deviation (SD) and sample size (N) of flowers in the field and plants in the common garden (garden) and the P-value from a linear model comparing the native and introduced range. Génova lacks parental population trait data for comparisons.

Population	Value	Corolla Length (mm)	Corolla Width (mm)	SA distance (mm)
Buffel's River (Introduced)	Field Mean, SD	34.38 ± 1.43	3.69 ± 0.56	0.74 ± 0.39
	Field N	45	45	45
	Garden Mean, SD	36.67 ± 1.92	6.09 ± 0.65	1.13 ± 0.43
	Garden N	635	632	623
	P value	**< 0.001*****	**0.005****	**< 0.001*****
Chayofa (Introduced)	Field Mean	34.07 ± 1.47	5.42 ± 0.53	1.15 ± 0.29
	Field N	167	167	168
	Garden Mean	34.88 ± 1.53	6.01 ± 0.44	1.19 ± 0.38
	Garden N	635	636	620
	P value	**< 0.001*****	**< 0.001*****	0.202
Piedra Pintada (Native)	Field Mean	38.64 ± 2.06	7.42 ± 0.91	1.18 ± 1.29
	Field N	45	45	45
	Garden Mean	38.94 ± 2.92	7.92 ± 0.78	1.18 ± 1.29
	Garden N	128	103	101
	P value	0.667	**0.022***	0.776
Tupiza (Native)	Field Mean	45.35 ± 3.06	8.01 ± 0.75	1.65 ± 1.81
	Field N	45	45	45
	Garden Mean	42.50 ± 3.38	8.46 ± 1.03	1.81 ± 0.81
	Garden N	245	198	226
	P value	**0.001****	**0.047***	**< 0.001*****
Génova (Introduced)	Garden Mean	36.46 ± 1.56	6.07 ± 0.46	0.96 ± 0.44
	Garden N	267	266	252

Table 6.3. Narrow Sense heritabilities of the floral traits, run separately for plants in the common garden sourced from Buffel's River and Chayofa. Included are the number of offspring and families (N), the slope (b), a 95% confidence interval for the slope, the heritability estimate and the P value showing significance of the regression.

Population of Origin	Trait	N	b	b 95% CI	h^2 estimate	P value
	Corolla Length	102 (12)	0.4272	0.05 – 0.80	0.85	0.055
Buffel's River	Corolla Width	102 (12)	0.1346	-0.18 – 0.45	0.27	0.432
	SA Distance	102 (12)	-0.0190	-0.33 – 0.30	-0.04	0.907
	Corolla Length	99 (14)	0.2775	0.05 – 0.50	0.16	**0.037**
Chayofa	Corolla Width	99 (14)	0.297	0.05 – 0.54	0.17	**0.038**
	SA Distance	98 (13)	0.449	0.06 – 0.84	0.29	**0.047**

6.4 Discussion

In this study we find low values of heritability for floral traits and thus evidence for plastic variation. We find significant but low heritabilities for all floral traits (corolla length, corolla width and SA distance) in one out of two populations studied, with the narrow sense heritability ranging from 0.16 to 0.27 but showing broad confidence intervals. Consistently, we found high phenotypic plasticity and large within-individual variation for SA distance, but not for the corolla traits. As expected, floral traits tended to show larger values in the common garden than in field populations and variation clustered more closely around a global mean in common garden plants than field population plants, indicating at least some environmental variation underlying the trait variation.

Our finding for the heritability of floral traits in *N. glauca* is that they can be significantly heritable, but are comparatively low when compared with typical findings of floral trait heritability studies. The narrow sense heritability of SA distance that we find ($h^2 = 0.29$) falls within the very broad expected range for this trait (0.20 – 0.85 from 17 estimates in 8 studies, Appendix Table A6.1; Opedal et al., 2017) but is low when compared with the range for other floral traits such as corolla length (see Appendix Table A3.2 in Chapter 3) and other floral trait groups (see Table S3 in Opedal, 2019). Although we could expect to find a high heritability of SA distance if selfing is the primary means of reproduction in a pollinator-absent population, our study suggests that floral traits in *N. glauca* are not strongly controlled by additive genetic variance. We also find no significant heritability of SA distance in plants sourced from a sunbird-

visited population in South Africa, with the significant result potentially being related to the pollinator-absent environment of the Tenerife population. Corolla length and corolla width show significant but low heritabilities in plants sourced from Tenerife, but this result is also not consistent across populations in the sample. Instead, floral traits in *N. glauca* are likely influenced primarily by environmental variation (see below).

There are potential limitations to our estimation of heritability with a low sample size, including the data harbouring a large degree of noise and large confidence intervals around the estimate. In addition, through parent offspring regression we can not control for maternal effects and parents and offspring were reared in different environments. Thus, further work on this study system could rear an additional generation so a sibling analysis could be used on the known pedigree for more reliable estimates of heritabilities. In addition, heritability in a fully selfing event may be more complicated and whilst limited, our correction for inbreeding can give a good indication of the heritability of the traits in that context.

We have evidence that the floral traits in our study are strongly influenced by environmental variation. For SA distance we find high plasticity (according to the RDPI metric) alongside with high within-individual variation across flowers, with up to 81.3% of the variation being within individuals in one population group in the common garden. Similarly, the field populations in Tenerife had a high percentage of variation (76.7%) at the within-individual level for this trait (Chapter 5). Our results suggest that SA distance can be more plastic than the corolla traits, with a high RDPI (mean = 0.221 ± 0.137; N = 33 plant pairs) that is surprising for a trait thought to be influential in reproductive assurance in populations where pollinators are absent. One interpretation for the reproductive success despite high plasticity of SA distance is that if the trait varies across flowers in all plants, then this allows the possibility of animal-mediated pollination when pollinators are present, whilst for plants in pollinator-absent areas a sufficient number of seeds are produced by selfing in flowers with shorter SA distances. It is expected that environmental variables such as temperature and rainfall can influence floral trait expression in plants (Brunet & Van Etten, 2019, Lambrecht et al., 2017).

Whilst we expected that non-native invasive populations could show higher plasticity of floral traits as compared with the native range populations (see Fig 6.1), our comparisons using the RDPI metric showed that only corolla length was more plastic in plants originating from native range populations, whilst corolla width and SA distance did not show differences. A high plasticity of floral traits related to reproduction (and therefore breeding system) is often advantageous for plants establishing in a new area (Cano et al., 2008; Peterson & Kay, 2015). As

described above, high variability in floral traits related to reproduction allow an alternate reproductive strategy to flourish in areas lacking pollinators. Further work could further investigate whether there is a relationship between invasion success and plasticity of floral traits in plants such as *N. glauca*.

We found that floral traits for plants grown in the common garden were often much larger and much less variable than their parental generation growing in wild populations. This further supports the idea that plants from these populations have a large environmental influence underlying the trait variation, since the plants show less difference when grown in the common environmental conditions. Even though this was the case, the comparison between plants in the common garden confirmed that the differences we see between ranges in corolla width in field populations (40.96% larger in the native populations; P = 0.023; N = 302) persist when grown in similar environmental conditions (36.89% larger in the native populations; P = 0.002; N = 1871). The morphological differentiation we find here for corolla width was not found in a study on *N. glauca* field populations by Garcia et al. (2020), whose PCA axis that correlated most strongly with corolla width did not show consistent differences between native and non-native populations. Narrower corollas could in theory increase the propensity of plants to self, as anthers and stigmas could be pushed closer together (Dart et al., 2012), but previous work found no evidence for a relationship between corolla width and the number of seeds produced by selfing (see Chapter 5). If future work confirmed that corollas are narrower in pollinator-absent non-native populations, then perhaps selection for wider corollas is relaxed in these populations.

6.4.1 Concluding remarks

In this study we find that whilst SA distances and corolla traits can be significantly heritable, the heritability is lower than expected for herkogamy and other traits closely related to reproductive success. This is surprising, since a trait so closely related to reproductive success would be expected to be under stronger genetic control. In addition, our study shows that environmental variation is likely a considerable factor in determining the variance of *N. glauca* floral traits, such as SA distance showing high phenotypic plasticity in all populations of origin studied. A high plasticity in floral traits and mating system favours invasiveness by providing plants the alternate option to set seed by selfing in the absence of pollinators (Goodwillie et al., 2010; Levin, 2010; Peterson & Kay, 2015). Future work could further investigate the role of plasticity in the invasion success of this species.

Chapter 7

General Discussion

7.1 Summary of findings

The aim of this thesis was to find the evolutionary and ecological consequences for plants following changes in their floral visitor assemblage. This included determining changes in the taxa that visit the plant, changes in the breeding system, how floral traits have changed between ranges, to what degree the floral trait variation is controlled by additive genetic variance and environmental variation and how reproductive output can be affected by changes in the visitor assemblage. Over the course of five data chapters I have used a combination of experimental and observational methodologies to study plant populations that show variation in the pollinator regime in order to address these aims, with each chapter described below.

I studied populations of *Digitalis purpurea* in their native and introduced ranges to test for differences in the visiting pollinator assemblages and subsequent ecological and evolutionary effects. I found that in the introduced populations, plants were still primarily relying on outcrossing to reproduce with hummingbirds visiting alongside bumblebees and both taxa depositing significantly numbers of pollen grains and thus being effective pollinators. This is in comparison to plants in the native range that are solely visited by two bumblebee species. Alongside changes in the pollinator taxa, in populations in Colombia and Costa Rica I found convergent changes towards larger proximal corolla tubes as compared with the native range populations in Britain. This floral trait restricts access to the nectaries, only allowing visitors with long mouthparts to reach the nectar. Therefore it was interesting to find strong and positive directional linear selection in introduced populations for this trait alongside the differences in morphology between ranges. A common garden experiment confirmed high heritability of this floral trait and others ($0.27 < h^2 < 0.49$), indicating a heritable basis of the trait for selection to act upon. A rapid evolutionary change in the proximal corolla tube would be consistent with new selective pressures expected following the addition of hummingbirds to the pollinating assemblage. The floral traits also appeared to be under at least some environmental control, as the variation expressed in the common garden clustered closer together around a global mean when plants were grown in the common garden and compared with variation in plants from field populations. I also find an indication of local adaptation of plants to their population of origin, since plants sourced from introduced populations produced smaller flowers in the common garden (whilst plants sourced from UK populations expressed higher trait means on average in the common garden). Considering the above, if plants in introduced populations show a similarly high heritability for floral traits such as the proximal corolla size, then the plants could still be responding to selection and evolving.

With the *D. purpurea* study system, I was able to test the effect of the addition of nectar robbers on the plant's reproductive output, since nectar robbers are a novel part of the floral visitor assemblage in introduced populations. In potted plants, I found that manually robbing flowers significantly reduced the rate of visitation and the length of visits by the two bumblebee species, *Bombus hortorum* and *B. pascuorum*, when compared with the control plants that were not manually robbed. Bumblebees also visited a smaller proportion of flowers on the robbed treatment plants, but there was no difference in the overall rate of rejection of inflorescences. Potentially owing to the above, plants that had undergone manual robbing produced significantly fewer seeds than control plants, although no difference was found for the number of pollen grains exported. These findings together indicate that nectar robbing can lead to less frequent and shorter visits by bumblebees, which in turn causes less effective pollination and thus fewer seeds produced by fruits.

I studied plants of *Nicotiana glauca* from wild populations and a common garden with plant sourced from various populations around the world. *N. glauca* flowers have adaptations for hummingbird pollination due to hummingbird visitation in the native range, but populations can vary in pollinator assemblage (if animal pollinated at all) and floral morphology. I first tested for a relationship between floral trait variation and the rate of seed production by selfing in wild plants in Tenerife populations, where we confirmed through a breeding system experiment that plants were relying on self-pollination due to a lack of suitable animal pollinators. I found a significant relationship between stigma-anther (SA) distance and selfing at the level of the flower in potted plants in the common garden, but no such relationship when testing at the level of the plant (with seed production of fruits averaged) in field populations. This could be due to the high level of phenotypic plasticity that I found for plants in field populations and the common garden, with up to 76.7% of the variation in stigma-anther distances being within individuals in field population plants, and 81.3% of the variation in SA distance being within individuals for plants grown in the common garden. Parent-offspring regression showed that whilst the floral traits were significantly heritable, the heritability range from 0.29 to 0.35 for SA distance. In addition, traits showed larger values in the common garden and clustered more closely together around a common mean (i.e. they varied less) than in the field. These results of low heritability of floral traits with a strong influence of environment variation could suggest decreased potential for a rapid response to selection if a shorter SA distance was favourable to increase the rate of selfing in pollinator-absent populations.

I also did not find any relationship between selfing and either of corolla length or corolla width, when tested at the plant level in field populations in Tenerife and in potted plants from the common garden.

The above results represent an original and interesting contribution to the field of plant evolutionary ecology, showing evolutionary and ecology changes following the addition and removal of floral visitors. In this final chapter, the findings presented in this thesis are discussed in the context of plant evolution whilst considering other studies from the literature.

7.2 Broader evolutionary context

The findings in this thesis contribute to an understanding that plants can evolve in response to new pollinator regimes and, in certain cases, have mechanisms to ensure reproductive success and persistence in areas lacking suitable pollinators. The new pollinator environments investigated involve shifts in the animal groups that deliver effective pollination for one study system and loss of animal pollination in some populations altogether for the other, with the latter involving a reliance on a new breeding system. In this section, I will tie the results from all five chapters on the two study systems together, using them to further discuss the interesting aspects of rapid floral evolution in response to global change and how plants can vary hugely in their floral traits.

Since studies showing rapid evolution of plant floral traits are rare (Campbell et al., 2018) my finding of rapid floral evolutionary innovation in *D. purpurea* indicates an interesting development within the field of plant evolutionary biology. The lack of studies is potentially due to the difficulty of detecting the often occasional episodes of rapid evolution that occur over a short time span (Hairston Jr et al., 2005). In Chapters on the *D. purpurea* study system, I found that the functional traits thought to be directly influencing access to the nectaries (larger proximal corolla tubes, particularly longer proximal corolla lengths), may be favoured in introduced populations by an improved efficiency of pollen transfer by the novel pollinators that includes species of hummingbirds. The introduced populations of *D. purpurea* also may have new selection pressures caused by the addition of non-mutualist nectar robbers that negatively affect reproductive output. In subsequent Chapters I investigated the *Nicotiana glauca* study system and focus on populations that have established in pollinator-absent areas and plants primarily reproduce by selfing. Both of these results taken together suggest that, in the face of changes in the distribution of plants and/or pollinator range changes and decline (Gómez-Ruiz & Lacher, 2019), plants may be able to cope with sudden changes in the pollinator regime and

subsequent novel selection (Gilman et al., 2012; Morton & Rafferty, 2017). In the case of *D. purpurea*, plants may have the ability to rapidly evolve corolla traits to accommodate morphology of animal taxa that are novel to the plant's pollinator environment. Further research would be required to assess the potential of plants to evolve or cope with sudden changes in the pollinator regime, such as an addition of a new functional pollinator group or removal of a functional group. In the case of non-native populations *N. glauca*, the plant relies on sexual reproduction by self-pollination when animal pollination is removed from the system, which may be facilitated by high plasticity of SA distance. If global change leads to removal of animal pollination from the environment of self-compatible plants, then there may be potential for plants to rely on selfing if there is enough plastic variation in the traits related to selfing. *N. glauca* is a very successful invasive species, which appears to be thriving in pollinator-absent populations despite the high levels of inbreeding that would be expected to be detrimental to survival (Charlesworth & Charlesworth, 1987).

Another interesting finding common to both study systems is the high degree to which the traits vary within individuals and within a family in the common garden experiments. Chapters on both study systems that assessed variation in floral traits in the field and common garden found that corolla traits varied highly within individuals and between individuals in a population. For example, I found that the amount of variation at the level of the individual was between 28.2% and 60.2% for the proximal corolla length and width in *Digitalis purpurea* and between 15.6% and 63.4% for corolla length and width in *Nicotiana glauca*. Since floral traits are typically involved in reproduction and pollination, it is interesting that they vary to a large degree plastically, but also even between individuals when grown in the similar environmental conditions of the common garden. It could also be expected that floral traits would be significantly and highly heritable due to their functional role, but this was not always the case. Whilst plasticity can expose new variation of traits to selection (Perry et al., 2018), high plasticity of traits can also help ensure survival and reproductive success when environmental variables are unpredictable (Leibman et al., 2018), such as in *N. glauca* when animal pollination becomes scarce. The degree to which floral traits vary at many levels of analysis in each study system would be an interesting aspect for further work.

Research into the two study systems has generated some interesting findings and implications for the potential of rapid floral evolution of floral traits, as well as for how selfing is controlled and how the floral traits can vary. There is more to discuss however, considering the avenues for further study with respect to limitations of the work.

7.3 Study limitations and future research

Although chapter 2 strongly suggests rapid evolution of larger proximal corolla tubes for easier access to nectar for hummingbirds, I do not yet provide direct evidence of this mechanism for selection by the hummingbirds. This could be tested for in the future with selective exclusion of pollinators, to compare lifetime seed production and pollen export between exclusive hummingbird visitation and exclusive bumblebee visitation regimes. Another test going beyond the effectiveness of hummingbird and bumblebee visits could also look at the preferences in animal groups for different floral morphologies. A comparison of plants between populations at the genomic level could also confirm whether there have been changes in allele frequencies between the ranges.

It is possible that populations studied in chapter 2 (and subsequently in chapter 3) are the same or part of an original population, with potential founder effects arising from the invasion process if only a subset of the original variation was brought into the new range. However, I show evidence that these populations are from independent introductions, from analysis of SNP markers showing they are genetically distinct from each other and each closer to those in the use (see methods of Chapter 2). Additionally, I find no difference in vegetative traits or other floral traits between range, which could be expected if plants differed from stochastic founder events. For the proximal and whole corolla traits, strong selection could potentially be decoupling the evolution of the two parts of the corolla, but further experimental work in introduced populations would be required to test this.

In chapter 3, I utilise a common garden experiment to study the expression of variation in plants from different parts of the range, in which I find evidence for local adaptation to their populations of origin. However useful, common garden experiments typically only provide a partial measurement of local adaptation (Nuismer & Gandon, 2008) and we assume high correlation between performance and floral trait size. Future work could grow plants from each population in both parts of the range in a reciprocal transplant experiment for a better test of local adaptation. Additionally, further study could test for plastic responses of plants to extreme environmental conditions, such as altering water availability or light levels, to find the limits of plasticity in the study system (Gallagher & Campbell, 2017; Leibman et al., 2018).

In my estimation of heritability using parent-offspring regression in chapter 3 (and later in chapter 6), issues could arise with comparisons between parents and offspring that developed in different environments. This includes underestimating h^2 due to the parents and offspring

developing in different environments and thus have more variation between them than would be expected if both generations were grown in similar environmental conditions (Conner et al., 2003; Kaczorowsi et al., 2008) but this is not an issue in all cases (Young et al., 1994). I also found difficulty in running a regression for population groups with too low a sample size, but this power problem was solved by running a regression for all plants in the common garden, across populations of origin. Despite these limitations, I was able to estimate heritabilities of traits in the common garden context using this method. Additionally with parent-offspring regressions, it is difficult to control for potential differences in maternal effects amongst parental plants. Future work crossing the plants and growing an F_2 generation would allow for a paternal half-sib (sibling) analysis that would control for maternal effects by reducing the number of mothers involved.

In chapter 4, several caveats limited the scope of the experimental work where I showed a robbing regime can lead to negative effects on reproductive success. The standardisation of the rate of visitation to the plants to 3 hours per day may not seem field realistic, but the number of seeds set by flowers on potted plants was consistent with the comparatively low seed set observed in plants from introduced populations. Additionally, I cannot rule out the possibility that manual robbing caused metabolic costs and resource depletion that contributes to a lower seed set than in control plants, so further experimentation could be used to distinguish between these effects and the effects that nectar robbers have (Doust & Doust, 1988; Navarro, 2001). It is also an issue that in this study, as in many others, that I use pollen export as a proxy to represent the male component of reproduction. This measurement does not fully reflect how many pollen grains reached stigmas on unrelated conspecific plants, so does not allow for the full picture of male fitness to be deduced. Future work on this study system could assess whether reproductive costs of robbing exist in natural populations in the introduced range, and whether hummingbird pollinators are dissuaded from visiting following detection of robbing holes (Lara & Ornelas, 2001) or from a lack of nectar (Irwin, 2000). In addition, it would be interesting to assess the potential role of nectar toxicity that could be discouraging visitation by nectar robbers in the native range (where none have been recorded to visit *D. purpurea*).

In chapters 5 and 6, I demonstrate that *Nicotiana glauca* flowers are more likely to self if stigma anther (SA) distances are shorter, but that individuals are highly plastic for this trait, so it is less surprising that I did not find a relationship at the level of the plant. I also did not find a relationship between other floral traits (corolla length and corolla width) at the level of the plant, and future work could test for a relationship between these traits and selfing at the level of

single flowers. Future work could also assess whether wind, or other abiotic and biotic factors, contribute to the variation in selfing in non-native populations.

In chapter 6 I found that corolla width showed significant differences between ranges, in both field populations and in the common garden experiment. If corollas are indeed narrower in pollinator-absent non-native populations, then further study could test whether selection for wide corollas in relaxed in these populations. Additionally in chapter 6, I find significant heritability of floral traits in the common garden plants, although the significance could be an artefact of population differences. However, I also find significant heritability of traits when testing for the non-native selfing population alone. Further work to confirm significant heritability of SA distance and other traits could rear an F_2 generation of plants so a paternal half-sib (sibling) analysis could be run to confirm the significant heritabilities whilst controlling for maternal effects. Further work on estimating the heritability of traits in the outcrossing populations should first estimate an inbreeding coefficient, since the flowers are self-compatible and it is only assumed that they are mostly outcrossing from hummingbird visitation.

7.4 Concluding remarks

Through original research presented in this thesis, I have contributed to a growing body of research that investigates the consequences for plants of altering pollinator environments and the assemblage of visitors. In five data chapters I have studied how plant systems cope with (i) the addition of novel functional pollinator taxa, (ii) the addition of an antagonistic interaction and (iii) removal of animal pollinators. Included in this thesis is the first study to show rapid evolution of a plant floral trait following an addition of a novel functional group of pollinators visiting the plant.

In chapters focussed on the *Digitalis purpurea* study system, I show that changes in the pollinator assemblage can lead to effects on reproductive output, a response to novel selection patterns and rapid evolution of floral traits. Changes in the visiting assemblage can include additions of pollinator mutualists that present a problem to plants with sub-optimal morphology, whilst additions of non-mutualists such as nectar robbers present a problem in the form of an antagonism, with associated negative effects on reproduction and fitness. In chapters focussing on *Nicotiana glauca* as a study system, I show how variation in the floral trait variation can be directly related to the number of seeds produced by selfing in flowers, but that a high within-individual variation for a trait can lead to no relationship with selfing being found at the plant level. This also implies how plants can benefit from plasticity in the trait variation

underlying the breeding system and thus flexibility of their mating strategy during colonisation of pollinator-scarce areas.

In this thesis, I demonstrate that relatively recent changes in the pollinator and visitor regime can be used to study how plants cope with sudden changes in their environment, including rapid evolutionary innovation. Novel interactions with mutualists and non-mutualists are becoming increasingly likely as human influence and other factors cause the ranges of both plants and animals to shift. It is important to understand the effects that new interacting partners can have on plant reproduction and their evolution, since interactions with pollinators has presumably been an important driving force in the diversification of angiosperm taxa. Studying how plants can cope with a sudden absence of animal pollination is also a useful tool to understand the flexibility of plant reproductive strategies in the face of pollinator decline.

References

Adler, L. S., Leege, L. M., & Irwin, R. E. (2016). Geographic variation in resistance to nectar robbing and consequences for pollination, *American Journal of Botany, 103*, 1819–1828.

Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C. J., & Koornneef, M. (1999). Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *96,* 4710-4717.

Alpert, P., & Simms, E. L., (2002). The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust?. *Evolutionary ecology*, *16*, 285-297.

Ameztegui, A. (2017). Plasticity: An R package to determine several plasticity indices. GitHub repository, https://github.com/ameztegui/Plasticity

Andalo, C., Burrus, M., Paute, S., Lauzeral, C., & Field, D.L. (2019). Prevalence of legitimate pollinators and nectar robbers and the consequences for fruit set in an *Antirrhinum majus* hybrid zone. *Botany Letters*, *166*, 80-92.

Anderson, B., Alexandersson, R., & Johnson, S. D. (2010). Evolution and co-existence of pollination ecotypes in an African *Gladiolus* (Iridaceae). *Evolution, 64*, 960-972.

Arbulo, N., Santos, E., Salvarrey, S., & Invernizzi, C. (2011). Proboscis length and resource utilization in two Uruguayan bumblebees: *Bombus atratus* Franklin and *Bombus bellicosus* Smith (Hymenoptera: Apidae). *Neotropical Entomology, 40*, 72–77.

Armbruster, W. S., Di Stilio, V. N. S., Tuxill, J.D., Flores, T. C., & Velasquez Runk, J. L. (1999). Covariance and decoupling of floral and vegetative traits in nine Neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *American Journal of botany*, *86*, 39-55.

Armbruster, W. S., Hansen, T. F., Pélabon, C., Pérez-Barrales, R., & Maad, J. (2009). The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Annals of botany*, *103*, 1529-1545.

Armbruster, W. S., Mulder, C. P., Baldwin, B. G., Kalisz, S., Wessa, B., & Nute, H. (2002). Comparative analysis of late floral development and mating-system evolution in tribe Collinsieae (Scrophulariaceae sl). *American Journal of Botany*, *89*, 37-49.

Ashman, T. L., & Majetic, C. J. (2006). Genetic constraints on floral evolution: A review and evaluation of patterns. *Heredity, 96*, 343–352.

Barlow, S. E., Wright, G. A., Ma, C., Barberis, M., Farrell, I. W., Marr, E. C., Brankin, A., Pavlik, B. M., & Stevenson, P. C. (2017). Distasteful nectar deters floral robbery. *Current Biology*, *27*, 2552-2558.

Barrett, S. C. (1998). The evolution of mating strategies in flowering plants. *Trends in plant science*, *3*, 335-341.

Barrett, S. C. H., Colautti, R. I., & Eckert, C. G. (2008). Plant reproductive systems and evolution during biological invasion. *Molecular Ecology, 17*, 373–383.

Barrett, R. D., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in ecology & evolution*, *23*, 38-44.

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1–48.

Berg, R. L. (1960). The Ecological Significance of Correlation Pleiades. *Evolution, 14*, 171–180.

Best, L. S., & Bierzychudek, P. (1982). Pollinator Foraging on Foxglove (*Digitalis purpurea*): A Test of a New Model. *Evolution, 36*, 70–79.

Bissell, E. K., & Diggle, P. K. (2010). Modular genetic architecture of floral morphology in *Nicotiana*: quantitative genetic and comparative phenotypic approaches to floral integration. *Journal of evolutionary biology*, *23*, 1744-1758.

Bodbyl Roels, S. A., & Kelly, J. K. (2011). Rapid evolution caused by pollinator loss in *Mimulus guttatus*. *Evolution, 65*, 2541–2552.

Bossdorf, O., Auge, H., Lafuma, L., Rogers, W. E., Siemann, E., & Prati, D. (2005). Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia*, *144,* 1-11.

Bozdogan, H. (1987). Model selection and Akaike's information criterion (AIC): The general theory and its analytical extensions. *Psychometrika*, *52*, 345-370.

Bräuchler, C., Meimberg, H., & Heubl, G. (2004). Molecular phylogeny of the genera *Digitalis* L. and *Isoplexis* (Lindley) Loudon (Veronicaceae) based on ITS-and trnL-F sequences. *Plant Systematics and Evolution, 248*, 111-128.

Broadbent, A., & Bourke, A. (2012). The bumblebee *Bombus hortorum* is the main pollinating visitor to *Digitalis purpurea* in a UK population. *Journal of Pollination Ecology, 8*, 48–51.

Bronstein, J. L., Barker J. L., Lichtenberg E. M., Richardson L. L., & Irwin, R. E. (2017). The behavioral ecology of nectar robbing: why be tactic constant? *Current Opinion in Insect Science, 21*, 14–18.

Brunet, J., & Eckert, C. G. (1998). Effects of floral morphology and display on outcrossing in blue columbine, *Aquilegia caerulea* (Ranunculaceae). *Functional Ecology*, *12,* 596-606.

Brunet, J., & Van Etten, M. L. (2019). The response of floral traits associated with pollinator attraction to environmental changes expected under anthropogenic climate change in high-altitude habitats. *International Journal of Plant Sciences*, *180*, 954-964.

Brys, R., Geens, B., Beeckman, T., & Jacquemyn, H. (2013). Differences in dichogamy and herkogamy contribute to higher selfing in contrasting environments in the annual *Blackstonia perfoliata* (Gentianaceae). *Annals of Botany*, *111*, 651-661.

Brys, R., & Jacquemyn, H. (2011). Variation in the functioning of autonomous self-pollination, pollinator services and floral traits in three *Centaurium* species. *Annals of Botany*, 107, 917-925.

Burkle L. A., Irwin R. E., & Newman D. A. (2007). Predicting the effects of nectar robbing on plant reproduction: Implications of pollen limitation and plant mating system. *American Journal of Botany*, *94*, 1935–1943.

Calle, J., Orjuela, N., & Moreno, E. (1989). Determinación de Gitoxina y Digitoxina en las hojas de *Digitalis purpurea* recolectada en dos sitios del departamento de Cundinamarca. *Revista Colombiana de Ciencias Químico-Farmacéuticas, 17*, 29–30.

Campbell, D. R. (1985). Pollen and gene dispersal: the influences of competition for pollination. *Evolution*, *39*, 418-431.

Campbell, D. R. (1996). Evolution of floral traits in a hermaphroditic plant: field measurements of heritabilities and genetic correlations. *Evolution*, *50*, 1442-1453.

Campbell, D. R., Faidiga, A., & Trujillo, G. (2018). Clines in traits compared over two decades in a plant hybrid zone. *Annals of Botany, 122*, 315–324.

Cano, L., Escarré, J., Fleck, I., Blanco-Moreno, J. M., & Sans, F. X. (2008). Increased fitness and plasticity of an invasive species in its introduced range: a study using *Senecio pterophorus. Journal of Ecology*, *96*, 468-476.

Carleial, S., Van Kleunen, M., & Stift, M. (2017). Small reductions in corolla size and pollen: ovule ratio, but no changes in flower shape in selfing populations of the North American *Arabidopsis lyrata*. *Oecologia*, *183*, 401-413.

Carr, D. E., & Fenster, C. B. (1994). Levels of genetic variation and covariation for *Mimulus* (Scrophulariaceae) floral traits. *Heredity*, *72*, 606-618.

Carrió, E., & Güemes J. (2019). Nectar robbing does not affect female reproductive success of an endangered *Antirrhinum* species, Plantaginaceae. *Plant Ecology and Diversity, 12*, 159–168.

Caruso, C. M. (2004). The quantitative genetics of floral trait variation in *Lobelia*: potential constraints on adaptive evolution. *Evolution*, *58*, 732-740.

Caruso, C. M., Eisen, K. E., Martin, R. A., & Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution, 73*, 4-14.

Castellanos, M. C., Montero-Pau, J., Ziarsolo, P., Blanca, J. M., Cañizares, J., & Pausas, J. G. (2019). A stable pollination environment limits current but not potential evolution of floral traits. *bioRxiv*, 581827.

Castellanos, M. C., Wilson, P., & Thomson, J. D. (2002). Dynamic nectar replenishment in flowers of *Penstemon* (Scrophulariaceae). *American Journal of Botany*, *89*, 111-118.

Castellanos, M. C., Wilson, P., & Thomson, J. D. (2003). Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution, 57,* 2742-2752.

Castro, S., Castro, M., Ferrero, V., Costa, J., Tavares, D., Navarro, L., & Loureiro, J. (2016). Invasion fosters change: independent evolutionary shifts in reproductive traits after *Oxalis pes-caprae* L. introduction. *Frontiers in plant science*, *7*, 874.

Castro S, Silveira P, & Navarro L. (2008). Consequences of nectar robbing for the fitness of a threatened plant species. *Plant Ecology 199,* 201–208.

Chalcoff, V. R., Lescano, M. N., & Devegili, A. M. (2019). Do novel interactions with local fauna have reproductive consequences for exotic plants? A case study with thistles, ants, aphids, and pollinators. *Plant Ecology*, 220, 125-134.

Charlesworth, D. & Charlesworth, B. (1987). Inbreeding depression and its evolutionary consequences. *Annual review of ecology and systematics*, *18*, 237-268.

Cheptou, P. O., Hargreaves, A. L., Bonte, D. & Jacquemyn, H. (2017). Adaptation to fragmentation: evolutionary dynamics driven by human influences. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*, 20160037.

Colautti, R. I., & Barrett, S. C. H. (2013). Rapid adaptation to climate facilitates range expansion of an invasive plant. *Science, 342*, 364–366.

Colautti, R. I., & Lau, J. A. (2015). Contemporary evolution during invasion: Evidence for differentiation, natural selection, and local adaptation. *Molecular Ecology, 24*, 1999–2017.

Colautti, R. I., Maron, J. L., & Barrett, S. C. (2009). Common garden comparisons of native and introduced plant populations: latitudinal clines can obscure evolutionary inferences. *Evolutionary applications*, *2*, 187-199.

Conner, J. K., Franks, R., & Stewart, C. (2003). Expression of additive genetic variances and covariances for wild radish floral traits: comparison between field and greenhouse environments. *Evolution*, *57*, 487-495.

Conner, J. K, Karoly, K., Stewart, C., Koelling, V. A., Sahli, H. F., & Shaw, F. H. (2011). Rapid independent trait evolution despite a strong pleiotropic genetic correlation. *American Naturalist, 178*, 429–441.

Cresswell, J. E. (2000). Manipulation of female architecture in flowers reveals a narrow optimum for pollen deposition. *Ecology*, *81*, 3244-3249.

Dart, S. R., Samis, K. E., Austen, E., & Eckert, C. G. (2012). Broad geographic covariation between floral traits and the mating system in *Camissoniopsis cheiranthifolia* (Onagraceae): multiple stable mixed mating systems across the species' range?. *Annals of botany*, *109*, 599-611.

Darwin, C. R. (1876). *The effects of cross and self fertilisation in the vegetable kingdom*. John Murray.

del Carmen Salas-Arcos, L., Lara, C., Castillo-Guevara, C., Cuautle, M., & Ornelas, J. F. (2019). "Pro-bird" floral traits discourage bumblebee visits to *Penstemon gentianoides* (Plantaginaceae), a mixed-pollinated herb. *The Science of Nature,* 106, 1.

del Castillo, R. C., & Fairbairn, D. J. (2012). Macroevolutionary patterns of bumblebee body size: detecting the interplay between natural and sexual selection. *Ecology and Evolution, 2*, 46–57.

Dellinger, A. S. (2020). Pollination syndromes in the 21st century: where do we stand and where may we go?. *New Phytologist*, 228, 1193-1213.

Descamps, C., Quinet, M., & Jacquemart, A. L. (2020). The effects of drought on plant–pollinator interactions: What to expect?. *Environmental and Experimental Botany*, 104297.

Díaz, G. J. (2011). Toxic plants of veterinary and agricultural interest in Colombia. *International Journal of Poisonous Plant Research, 1*, 1-19.

Ding, B., Mou, F., Sun, W., Chen, S., Peng, F., Bradshaw, H. D., & Yuan, Y. W. (2017). A dominant-negative actin mutation alters corolla tube width and pollinator visitation in *Mimulus lewisii*. *New Phytologist, 213*, 1936–1944.

Dole, J. A. (1990). Role of corolla abscission in delayed self-pollination of *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany*, 77, 1505-1507.

dos Santos, J. M. A., Carneiro, L. T., & Martins, C. F. (2020). Are native nectar robbers against the alien? Effects of floral larceny on the reproductive success of the invasive yellow bells (*Tecoma stans*, Bignoniaceae). *Acta Oecologica*, 105, 103547.

Doust, J. L., & Doust, L. L. eds. (1988). Plant reproductive ecology: patterns and strategies. Oxford University Press on Demand.

Eckert, C. G., & Schaefer, A. (1998). Does self-pollination provide reproductive assurance in *Aquilegia canadensis* (Ranunculaceae)?. *American Journal of Botany*, 85, 919-924.

Elle, E. (1998). The quantitative genetics of sex allocation in the andromonoecious perennial, *Solanum carolinense* (L.). *Heredity*, 80, 481-488.

Elzinga, J. A., Atlan, A., Biere, A., Gigord, L., Weis, A. E., & Bernasconi, G. (2007). Time after time: flowering phenology and biotic interactions. *Trends in ecology & evolution*, 22, 432-439.

Evans, F. J., & Cowley, P. S. (1972). Cardenolides and spirostanols in *Digitalis purpurea* at various stages of development. *Phytochemistry*, 11, 2971-2975.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., & Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics, 35*, 375–403.

Fenster, C. B., & Carr, D. E. (1997). Genetics of sex allocation in *Mimulus* (Scrophulariaceae). *Journal of Evolutionary Biology*, *10*, 641-661.

Fernandez, G. C. J., & Miller, J.C. (1985). Estimation of heritability by parent-offspring regression. *Theoretical and Applied Genetics*, *70*, 650-654.

Ferrero, V., Navarro, L., Castro, S., Loureiro, J., Sánchez, J. M., Carvallo, G. O., & Barrett, S. C. H. (2020). Global patterns of reproductive and cytotype diversity in an invasive clonal plant. *Biological Invasions, 9*, 1–13.

Fishman, L., Kelly, A. J., & Willis, J. H. (2002). Minor quantitative trait loci underlie floral traits associated with mating system divergence in *Mimulus*. *Evolution*, *56*, 2138-2155.

Florentine, S. K., Westbrooke, M. E., Gosney, K., Ambrose, G., & O'Keefe, M. (2006). The arid land invasive weed *Nicotiana glauca* R. Graham (Solanaceae): Population and soil seed bank dynamics, seed germination patterns and seedling response to flood and drought. *Journal of Arid Environments*, 66, 218-230.

Frankel, O. H., Brown, A. H., & Burdon, J. J. (1995). *The conservation of plant biodiversity*. Cambridge University Press.

Franks, S. J., & Weis, A. E. (2008). A change in climate causes rapid evolution of multiple life-history traits and their interactions in an annual plant. *Journal of evolutionary biology*, *21*, 1321-1334.

Furer, V., Hersch, M., Silvetzki, N., Breuer, G. S., & Zevin, S. (2011). *Nicotiana glauca* (tree tobacco) intoxication—two cases in one family. *Journal of medical toxicology*, *7*, 47-51.

Gaffal, K. P., Heimler, W., & El-Gammal, S. (1998). The floral nectary of *Digitalis purpurea* L., structure and nectar secretion. *Annals of Botany*, *81*, 251-262.

Galen, C. (1999). Why do flowers vary? The functional ecology of variation in flower size and form within natural plant populations. *Bioscience*, *49*, 631-640.

Galen, C., & Cuba, J. (2001). Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution*, *55*, 1963-1971.

Gallagher, M. K., & Campbell, D. R. (2017). Shifts in water availability mediate plant–pollinator interactions. *New Phytologist*, *215*, 792-802.

García, M., Benítez-Vieyra, S., Sérsic, A. N., Pauw, A., Cocucci, A. A., Traveset, A., Sazatornil, F., & Paiaro, V. (2020). Is variation in flower shape and length among native and non-native populations of *Nicotiana glauca* a product of pollinator-mediated selection?. *Evolutionary Ecology*, *34*, 893-913.

Geber, M. A., & Griffen, L. R. (2003). Inheritance and natural selection on functional traits. *International journal of plant sciences*, *164*, S21-S42.

Geerts, S., & Pauw, A. (2009). African sunbirds hover to pollinate an invasive hummingbird-pollinated plant. *Oikos*, *118*, 573-579.

Gervasi, D. D. L., & Schiestl, F. P. (2017). Real-time divergent evolution in plants driven by pollinators. *Nature Communications, 8*, 1–8.

Gibson, P. T. (1996). Correcting for Inbreeding in Parent-Offspring Regression Estimates of Heritability with Non-Additive and Genotype × Environment Effects Present. *Crop science*, *36,* 594-600.

Gilman, R. T., Fabina, N. S., Abbott, K. C. & Rafferty, N. E. (2012). Evolution of plant–pollinator mutualisms in response to climate change. *Evolutionary Applications*, *5*, 2-16.

Gómez-Ruiz, E. P. & Lacher Jr, T. E. (2019). Climate change, range shifts, and the disruption of a pollinator-plant complex. *Scientific reports*, *9*, 1-10.

González-Varo, J. P., Biesmeijer, J. C., Bommarco, R., Potts, S. G., Schweiger, O., Smith, H. G., Steffan-Dewenter, I., Szentgyörgyi, H., Woyciechowski, M., & Vilà, M. (2013). Combined effects of global change pressures on animal-mediated pollination. *Trends in Ecology and Evolution*, *28*, 524-530.

Goodspeed, T. H. (1954). The Genus *Nicotiana*. Origins, Relationships and Evolution of Its Species in the Light of Their Distribution, Morphology and Cytogenetics; Chronica Botanica Company, Waltham, MA, USA, 1954, and Stechert-Hafner. Inc., New York, NY, USA.

Goodwillie, C., Sargent, R. D., Eckert, C. G., Elle, E., Geber, M. A., Johnston, M. O., Kalisz, S., Moeller, D. A., Ree, R. H., Vallejo-Marin, M., & Winn, A. A. (2010). Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist*, *185*, 311-321.

Goodwillie, C., & Weber, J. J. (2018). The best of both worlds? A review of delayed selfing in flowering plants. *American Journal of Botany*, *105*, 641-655.

Goulson, D. Cruise, J. L. Sparrow, K. R., Harris, A. J. Park, K. J. Tinsley, M. C., & Gilburn, A. S. (2007). Choosing rewarding flowers; perceptual limitations and innate preferences influence decision making in bumblebees and honeybees. *Behavioural Ecology and Sociobiology, 61*, 1523-1529.

Goulson, D., & Darvill. B. (2004). Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? *Apidologie, 35*, 55–63.

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science, 347*, 6229.

Grant, K. A., & Grant, V. (1968). *Hummingbirds and their flowers*. Columbia University Press.

Grant, P. R., & Grant, B. R. (1992). Demography and the genetically effective sizes of two populations of Darwin's finches. *Ecology*, *73*, 766-784.

Grant, P. R., & Grant, B. R. (1995). Predicting microevolutionary responses to directional selection on heritable variation. *Evolution, 49*, 241-251.

Grant, V., & Grant, K. A. (1965). *Flower Pollination in the Phlox Family*. Columbia University Press.

Grass, I., Berens, D. G., & Farwig, N. (2014). Natural habitat loss and exotic plants reduce the functional diversity of flower visitors in a heterogeneous subtropical landscape. *Functional Ecology, 28*, 1117–1126.

Grindeland, J. M. (2008). Inbreeding depression and outbreeding depression in *Digitalis purpurea*: optimal outcrossing distance in a tetraploid. *Journal of evolutionary biology*, *21*, 716-726.

Grindeland, J. M., Sletvold, N., & Ims, R. A. (2005). Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. *Functional Ecology, 19*, 383–390.

Hairston, N. G. & Dillon, T. A. (1990). Fluctuating selection and response in a population of freshwater copepods. *Evolution*, *44*, 1796-1805.

Hairston Jr, N. G., Ellner, S. P., Geber, M. A., Yoshida, T. & Fox, J. A. (2005). Rapid evolution and the convergence of ecological and evolutionary time. *Ecology letters*, *8*, 1114-1127.

Hansen, T. F., Pélabon, C., Armbruster, W. S. & Carlson, M. L. (2003). Evolvability and genetic constraint in *Dalechampia* blossoms: components of variance and measures of evolvability. *Journal of evolutionary biology*, *16*, 754-766.

Hansen, T.F., Pélabon, C. & Houle, D. (2011). Heritability is not evolvability. *Evolutionary Biology*, *38,* 258-277.

Harder, L. D., & Johnson, S. D. (2009). Darwin's beautiful contrivances: Evolutionary and functional evidence for floral adaptation. *New Phytologist, 183*, 530–545.

Hartfield, M. (2016). Evolutionary genetic consequences of facultative sex and outcrossing. *Journal of evolutionary biology*, 29, 5-22.

Heiling J. M., Ledbetter T. A., Richman S. K., Ellison H. K., Bronstein J. L., & Irwin R. E. (2018). Why are some plant–nectar robber interactions commensalisms? *Oikos, 127*, 1679–1689.

Heinrich, B. (1979). Resource heterogeneity and patterns of movement in foraging bumblebees. *Oecologia*, *40*, 235-245.

Herlihy, C. R. & Eckert, C. G. (2007). Evolutionary analysis of a key floral trait in Aquilegia canadensis (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution: International Journal of Organic Evolution*, *61*, 1661-1674.

Herrera, C. M., Castellanos, M. C., & Medrano, M. (2006). Geographical context of floral evolution: towards an improved research programme in floral diversification. In: L. D. Harder

and S. C. H. Barrett (Eds.) *The ecology and evolution of flowers* (278–294). Oxford University Press,

Herrera, C. M., & Pellmyr, O. eds. (2009). *Plant animal interactions: an evolutionary approach*. John Wiley & Sons.

Heywood, V. H. (1951). The distribution of the *Digitalis purpurea* complex. In: J. E. Lousley (Ed.) *The distribution of British plants – Report of the conference held in 1950 by the Botanical Society of the British Isles* (104-105). T. Bungle and Co.

Higashi, S., Ohara, M., Arai, H. & Matsuo, K. (1988). Robber-like pollinators: overwintered queen bumblebees foraging on *Corydalis ambigua*. *Ecological Entomology*, *13*, 411-418.

Hodges, C. M. & Wolf, L. L. (1981). Optimal foraging in bumblebees: why is nectar left behind in flowers?. *Behavioral Ecology and Sociobiology*, *9*, 41-44.

Holland, J. B., Nyquist, W. E. & Cervantes-Martínez, C. T. (2003). Estimating and interpreting heritability for plant breeding: an update. *Plant breeding reviews*, 22, 9-112.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal, 50*, 346–363.

Inouye, D. W. (1983). The ecology of nectar robbing. *The biology of nectaries*, 153-173.

Irwin, R. E. (2000). Hummingbird avoidance of nectar-robbed plants: spatial location or visual cues. *Oikos*, 91, 499-506.

Irwin, R. E., & Brody A. K. (1999). Nectar-robbing bumble bees reduce the fitness of *Ipomopsis aggregata* (Polemoniaceae). *Ecology, 80*, 1703–1712.

Irwin, R. E., Bronstein, J. L., Manson, J. S., & Richardson, L. (2010). Nectar Robbing: Ecological and Evolutionary Perspectives. *Annual Review of Ecology, Evolution, and Systematics, 41*, 271–292.

Irwin, R. E., Howell, P. & Galen, C. (2015). Quantifying direct vs. indirect effects of nectar robbers on male and female components of plant fitness. *Journal of Ecology*, 103, 1487-1497.

Irwin, R. E., & Maloof, J.E. (2002). Variation in nectar robbing over time, space, and species. *Oecologia, 133*, 525–533.

Issaly, E. A, Sérsic, A. N., Pauw, A., Cocucci, A. A., Traveset, A., Benítez-Vieyra, S. M., and Paiaro, V. (2020). Reproductive ecology of the bird-pollinated *Nicotiana glauca* across native and introduced ranges with contrasting pollination environments. *Biological Invasions, 22*, 485–498.

Jarne, P., & Charlesworth, D., (1993). The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annual Review of Ecology and Systematics*, *24*, 441-466.

Jiang, X. F., Zhu, X. F., & Li, Q. J. (2018). Variation in the degree of reciprocal herkogamy affects the degree of legitimate pollination in a distylous species. *AoB Plants*, *10*, ply022.

Joshi, J., Schmid, B., Caldeira, M. C., Dimitrakopoulos, P. G., Good, J., Harris, R., Hector, A., Huss-Danell, K., Jumpponen, A., Minns, A., & Mulder, C. P. H. (2001). Local adaptation enhances performance of common plant species. *Ecology Letters*, *4*, 536-544.

Kaczorowski, R. L., Juenger, T. E., & Holtsford, T. P. (2008). Heritability and correlation structure of nectar and floral morphology traits in *Nicotiana alata*. *Evolution: International Journal of Organic Evolution*, *62*, 1738-1750.

Kalisz, S., & Vogler, D. W. (2003). Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology*, *84,* 2928-2942.

Kalisz, S., Vogler, D., Fails, B., Finer, M., Shepard, E., Herman, T., & Gonzales, R. (1999). The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). *American Journal of Botany*, *86*, 1239-1247.

Kawecki, T. J. & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology letters*, *7*, 1225-1241.

Krauss, S. L, Phillips, R. D., Karron, J. D., Johnson, S. D., Roberts, D. G., & Hopper, S. D. (2017). Novel consequences of bird pollination for plant mating. *Trends in Plant Science, 22*, 395-410.

Kudo, G. (2003). Anther arrangement influences pollen deposition and removal in hermaphrodite flowers. *Functional Ecolo*gy, *17*, 349-355.

Kulbaba, M. W., & Worley, A. C. (2008). Floral design in *Polemonium brandegei* (Polemoniaceae): genetic and phenotypic variation under hawkmoth and hummingbird pollination. *International Journal of Plant Sciences*, *169*, 509-522.

Kwak, M. M. (1978). Pollination, hybridization and ethological isolation of *Rhinanthus minor* and *R. serotinus* (Rhinanthoideae: Scrophulariaceae) by bumblebees (*Bombus* Latr.). *Taxon*, 145-158.

Lambrecht, S. C., Morrow, A., & Hussey, R. (2017). Variation in and adaptive plasticity of flower size and drought-coping traits. *Plant Ecology*, *218*, 647-660.

Lande, R., & Arnold, S. J. (1983). The measurement of selection on correlated characters. *Evolution, 37*, 1210-1226.

Lara, C., & Ornelas, J. (2001). Preferential nectar robbing of flowers with long corollas: Experimental studies of two hummingbird species visiting three plant species. *Oecologia*, *128*, 263–273.

Lehtilä, K., & Holmén Bränn, K. (2007). Correlated effects of selection for flower size in *Raphanus raphanistrum*. *Canadian Journal of Botany, 85*, 160–166.

Leibman, L., Rowe, A., Koski, M. H. & Galloway, L. F. (2018). Populations with greater flexibility in floral traits modify mating system in response to the pollinator environment. *Functional Ecology*, *32*, 1457-1466.

Lendvai, G., & Levin, D. A. (2003). Rapid response to artificial selection on flower size in *Phlox*. *Heredity, 90*, 336–342.

Lenth, R., Singmann, H., Love, J., Buerkner, P., & Herve, M. (2018). Emmeans: Estimated marginal means, aka least-squares means. R package version, *1*, 3.

Levin, D. A. (2010). Environment-enhanced self-fertilization: Implications for niche shifts in adjacent populations. *Journal of Ecology*, *98*, 1276-1283.

Li, D. F., Yan, X. C., Lin, Y., Wang, L., & Wang, Q. (2021). Do flowers removed of either nectar or pollen attract fewer bumblebee pollinators? An experimental test in *Impatiens oxyanthera*. *AoB Plants*.

Liedl, B. E., & Anderson, N. O. (1993). Reproductive barriers: identification, uses and circumvention. *Plant Breed Rev*, *11*, 11-154.

Lloyd, D. G. & Webb, C.J. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand journal of botany*, *24*, 135-162.

Lortie, C. J., & Hierro, J. L. (2021). A synthesis of local adaptation to climate through reciprocal common gardens. *Journal of Ecology*.

Lustenhouwer, N., Wilschut, R. A., Williams, J. L., van der Putten, W. H., & Levine, J. M. (2018). Rapid evolution of phenology during range expansion with recent climate change. *Global Change Biology, 24*, 534–544.

Lynch, M., & Walsh, B., (1998). Genetics and analysis of quantitative traits.

Mackin, C. R., Peña, J. F., Blanco, M. A., Balfour, N. J., & Castellanos, M. C., (2021). Rapid evolution of a floral trait following acquisition of novel pollinators. *Journal of Ecology*, *109*, 2234-2246.

Mal, T. K., & Lovett-Doust, J. (2005). Phenotypic plasticity in vegetative and reproductive traits in an invasive weed, *Lythrum salicaria* (Lythraceae), in response to soil moisture. *American Journal of Botany*, *92*, 819-825.

Maloof, J. E. (2001). The effects of a bumble bee nectar robber on plant reproductive success and pollinator behavior. *American Journal of Botany*, *88*, 1960-1965.

Maloof, J. E., & Inouye, D.W. (2000). Are nectar robbers cheaters or mutualists? *Ecology*, *81*, 2651-2661.

Manning, A. (1956). Some aspects of the foraging behaviour of bumble-bees. *Behaviour, 9*, 164–201.

Maron, J. L., Vilà, M., Bommarco, R., Elmendorf, S., & Beardsley, P. (2004). Rapid evolution of an invasive plant. *Ecological Monographs*, *74*, 261-280.

Mitchell, R. J., & Shaw, R. G. (1993). Heritability of floral traits for the perennial wild flower *Penstemon centranthifolius* (Scrophulariaceae): clones and crosses. *Heredity*, *71*, 185-192.

Morris, W. F. (1996). Mutualism denied? Nectar-robbing bumble bees do not reduce female or male success of bluebells. *Ecology*, *77*, 1451-1462.

Morrissey, M. B., Kruuk, L. E. B., & Wilson, A. J. (2010). The danger of applying the breeder's equation in observational studies of natural populations. *Journal of evolutionary biology*, *23*, 2277-2288.

Morrissey, M. B., & Sakrejda, K. (2013). Unification of Regression-Based Methods for the Analysis of Natural Selection. *Evolution, 67*, 2094–2100.

Morrissey, M., & Sakrejda, K. (2014). gsg: Calculation of selection coefficients. R package version 2.0.

Morton, E. M. & Rafferty, N. E. (2017). Plant–pollinator interactions under climate change: The use of spatial and temporal transplants. *Applications in plant sciences*, *5*, 1600133.

Motten, A. F., & Stone, J. L. (2000). Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany*, 87(3), pp.339-347.

Murúa, M., Espinoza, C., Bustamante, R., Marín, V. H., & Medel, R. (2010). Does human-induced habitat transformation modify pollinator-mediated selection? A case study in *Viola portalesia* (Violaceae). *Oecologia, 163*, 153–162.

Nakagawa, S., & Schielzeth, H. (2013). A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods in ecology and evolution*, *4*, 133-142.

Nattero, J., & Cocucci, A. A. (2007). Geographical variation in floral traits of the tree tobacco in relation to its hummingbird pollinator fauna. *Biological Journal of the Linnean Society*, *90*, 657-667.

Nattero, J., Sérsic, A. N., & Cocucci, A. A. (2010). Patterns of contemporary phenotypic selection and flower integration in the hummingbird-pollinated *Nicotiana glauca* between populations with different flower-pollinator combinations. *Oikos, 119*, 852–863.

Nattero, J., Sérsic, A. N., & Cocucci, A. A. (2011). Geographic variation of floral traits in *Nicotiana glauca*: relationships with biotic and abiotic factors. *Acta Oecologica, 37*, 503–511.

Navarro, L. (2001). Reproductive biology and effect of nectar robbing on fruit production in *Macleania bullata* (Ericaceae). *Plant Ecology*, *152*, 59-65.

Navarro, L., & Medel, R. (2009). Relationship between floral tube length and nectar robbing in *Duranta erecta* L.(Verbenaceae). *Biological Journal of the Linnean Society*, *96*, 392-398.

Nazir, R., Reshi, Z., & Wafai, B. A. (2008). Reproductive ecology of medicinally important Kashmir Himalayan species of *Digitalis* L. *Plant Species Biology, 23*, 59–70.

Ne'eman, G., Jürgens, A., Newstrom-Lloyd, L., Potts, S. G., & Dafni, A. (2010). A framework for comparing pollinator performance: effectiveness and efficiency. *Biological Reviews*, *85*, 435-451.

Newman, E., Manning, J., & Anderson, B. (2015). Local adaptation: Mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution*, *69*, 2262-2275.

Nuismer, S. L., & Gandon, S. (2008). Moving beyond common-garden and transplant designs: insight into the causes of local adaptation in species interactions. *The American Naturalist*, *171*, 658-668.

Nyquist, W. E., & Baker, R. J. (1991). Estimation of heritability and prediction of selection response in plant populations. *Critical reviews in plant sciences*, *10*, 235-322.

Oduor, A. M., Leimu, R., & van Kleunen, M. (2016). Invasive plant species are locally adapted just as frequently and at least as strongly as native plant species. *Journal of Ecolog*y, *104*, 957-968.

Ollerton, J., Alarcón, R., Waser, N. M., Price, M. V., Watts, S., Cranmer, L., Hingston, A., Peter, C. I., & Rotenberry, J. (2009). A global test of the pollination syndrome hypothesis. *Annals of botany*, *103*, 1471-1480.

Ollerton, J., Watts, S., Connerty, S., Lock, J., Parker, L., Wilson, I., Schueller, S., Nattero, J., Cocucci, A. A., Izhaki, I., & Geerts, S. (2012). Pollination ecology of the invasive tree tobacco *Nicotiana glauca*: comparisons across native and non-native ranges. *Journal of Pollination Ecology*, *9*.

Opedal, Ø. H. (2018). Herkogamy, a principal functional trait of plant reproductive biology. *International Journal of Plant Sciences*, *179*, 677-687.

Opedal, Ø. H. (2019). The evolvability of animal-pollinated flowers: towards predicting adaptation to novel pollinator communities. *New Phytologist*, *221*, 1128-1135.

Opedal, Ø. H., Albertsen, E., Armbruster, W. S., Pérez-Barrales, R., Falahati-Anbaran, M., & Pélabon, C. (2016). Evolutionary consequences of ecological factors: pollinator reliability predicts mating-system traits of a perennial plant. *Ecology Letters*, *19*, 1486-1495.

Opedal, Ø. H., Bolstad, G. H., Hansen, T. F., Armbruster, W. S., Pélabon, C. (2017). The evolvability of herkogamy: Quantifying the evolutionary potential of a composite trait. *Evolution*, *71*, 1572-1586.

Ørsted, A. S. (1863). *L'Amérique Centrale: Recherches sur sa flora et sa géographie physique. Résultats d'un voyage dans les états de Costa Rica et de Nicaragua exécuté pendant les années 1846–1848*. B. Luno par FS Muhle.

Palmer-Young, E. C., Farrell, I. W., Adler, L. S., Milano, N. J., Egan, P. A., Irwin, R. E., & Stevenson, P. C. (2019). Secondary metabolites from nectar and pollen: a resource for ecological and evolutionary studies. *Ecology, 100*, e02621. 10.1002/ecy.2621

Pannell, J. R. (2015). Evolution of the mating system in colonizing plants. *Molecular Ecology*, *24*, 2018-2037.

Parachnowitsch, A. L., Manson, J. S., & Sletvold, N. (2019). Evolutionary ecology of nectar. *Annals of Botany, 123*, 247–261.

Paradis, E., & Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, *35*, 526-528.

Paudel, B. R., Shrestha, M., Burd, M., Adhikari, S., Sun, Y. S., & Li Q. J. (2016). Coevolutionary elaboration of pollination-related traits in an alpine ginger (*Roscoea purpurea*) and a tabanid fly in the Nepalese Himalayas. *New Phytologist, 211*, 1402-1411.

Pauw, A. (2019). A Bird's-Eye View of Pollination: Biotic Interactions as Drivers of Adaptation and Community Change. *Annual Review of Ecology, Evolution, and Systematics, 50*, 477-502.

Percival, M., & Morgan, P. (1965). Observations on the floral biology of *Digitalis* species. *New Phytologist*, *64*, 1-22.

Pérez-Arbeláez, E. *Plantas útiles de Colombia*. (1978). Bogotá: Litografía Arco.

Pérez-Barrales, R., Arroyo, J., & Armbruster, S. W. (2007). Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae). *Oikos, 116*, 1904–1918.

Perry, B. W., Schield, D. R. & Castoe, T. A. (2018). Evolution: Plasticity versus selection, or plasticity and selection? *Current Biology*, *28*, R1104-R1106.

Petanidou, T., Godfree, R. C., Song, D. S., Kantsa, A., Dupont, Y. L., & Waser, N. M. (2012). Self-compatibility and plant invasiveness: Comparing species in native and invasive ranges. *Perspectives in Plant Ecology, Evolution and Systematics, 14*, 3–12.

Peterson, B. G., Carl, P., Boudt, K., Bennett, R., Ulrich, J., Zivot, E., Cornilly, D., Hung, E., Lestel, M., Balkissoon, K., & Wuertz, D. (2018). Package 'performanceanalytics'. R Team Cooperation.

Peterson, M. L., & Kay, K. M. (2015). Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist*, *185*, 28-43.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., & Maintainer, R. (2017). Package 'nlme'. Linear and nonlinear mixed effects models, version, *3*.

Poorter, H., Fiorani, F., Stitt, M., Schurr, U., Finck, A., Gibon, Y., Usadel, B., Munns, R., Atkin, O. K., Tardieu, F., & Pons, T. L. (2012). The art of growing plants for experimental purposes: a practical guide for the plant biologist. *Functional Plant Biology*, *39*, 821-838.

Pyke, G. H. (1991). What does it cost a plant to produce floral nectar?. *Nature*, *350*, 58-59.

Qu, R., Li, X., Luo, Y., Dong, M., Xu, H., Chen, X., & Dafni, A. (2007). Wind-dragged corolla enhances self-pollination: a new mechanism of delayed self-pollination. *Annals of Botany*, *100*, 1155-1164.

R Core Team. (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Razanajatovo, M., Fischer, L., & van Kleunen, M. (2020). Do floral traits and the selfing capacity of *Mimulus guttatus* plastically respond to experimental temperature changes?. *Oecologia*, *192*, 261-272.

Reinig, W. F., & Rasmont, P. (1988). Beitrag zur Kenntnis der Bergwaldhummel *Alpigenobombus wurfleini* (Radoszkowski, 1859) (Hymenoptera, Apidae, Bombinae). *Spixiana*, *11*, 37-67.

Reznick, D. N., & Ricklefs, R. E. (2009). Darwin's bridge between microevolution and macroevolution. *Nature*, *457*, 837-842.

Richardson, S. C. (2004). Are nectar-robbers mutualists or antagonists?. *Oecologia*, *139*, 246-254.

Richardson, D. M., Allsopp, N., D'Antonio, C. M., Milton, S. J., & Rejmánek, M. (2000). Plant invasions–the role of mutualisms. *Biological Reviews*, *75*, 65-93.

Richman, S. K., Irwin, R. E., Bosak, J. T., & Bronstein, J. L. (2018). Consequences of secondary nectar robbing for male components of plant reproduction. *American Journal of Botany*, *105*, 943–949.

Ritland, K., & Ritland, C. (1996). Inferences about quantitative inheritance based on natural population structure in the yellow monkeyflower, *Mimulus guttatus. Evolution*, *50*, 1074-1082.

Riveros, A. J., Hernandez, E. J., & Nates-Parra, G. (2006). Morphological constraints and nectar robbing in three Andean bumble bee species (Hymenoptera, Apidae, *Bombini*). *Caldasia, 28*, 111–114.

Rojas-Nossa, S. V., Sanchez, J., & Navarro, L. (2016). Nectar robbing: a common phenomenon mainly determined by accessibility constraints, nectar volume and density of energy rewards. *Oikos*. *125*, 1044-1055.

Rojas-Nossa, S. V., Sánchez, J. M., & Navarro, L. (2021). Nectar robbing and plant reproduction: an interplay of positive and negative effects. *Oikos*, *130*, 601-608.

Salamin, N., Wüest, R. O., Lavergne, S., Thuiller, W., & Pearman, P. B. (2010). Assessing rapid evolution in a changing environment. *Trends in ecology & evolution*, *25*, 692-698.

Schueller, S. K. (2004). Self-pollination in island and mainland populations of the introduced hummingbird-pollinated plant*, Nicotiana glauca* (Solanaceae). *American Journal of Botany*, *91*, 672-681.

Schueller, S. K. (2007). Island-mainland difference in *Nicotiana glauca* (Solanaceae) corolla length: A product of pollinator-mediated selection? *Evolutionary Ecology, 21*, 81–98.

Schwaegerle, K. E., & Levin, D. A. (1991). Quantitative genetics of fitness traits in a wild population of Phlox. *Evolution*, *45*, 169-177.

Shore, J. S., & Barrett, S. C. (1990). Quantitative genetics of floral characters in homostylous *Turnera ulmifolia* var. *angustifolia* Willd. (Turneraceae). *Heredity*, *64*, 105-112.

Smith, J. D., & Kinman, M. L. (1965). The Use of Parent-Offspring Regression as an Estimator of Heritability. *Crop Science*, *5*, 595-596.

Southwick, E. E. (1984). Photosynthate allocation to floral nectar: a neglected energy investment. *Ecology*, *65*, 1775-1779.

Souza, C. V. D., Salvador, M. V., Tunes, P., Di Stasi, L. C., & Guimarães, E. (2019). I've been robbed! – Can changes in floral traits discourage bee pollination?. *Plos one*, *14*, e0225252.

Stebbins, G. L. (1970). Adaptive Radiation of Reproductive Characteristics in Angiosperms, I: Pollination Mechanisms. *Annual Review of Ecology and Systematics, 1*, 307–326.

Stinchcombe, J. R., Agrawal, A. F., Hohenlohe, P., Arnold, S. J., and Blows, M. W. (2008). Estimating nonlinear selection gradients using quadratic regression coefficients: double or nothing? *Evolution, 62*, 2435–2440.

Stout J. C., Allen J. A., & Goulson D. (2000). Nectar robbing, forager efficiency and seed set: Bumblebees foraging on the self incompatible plant *Linaria vulgaris* (Scrophulariaceae). *Acta Oecologica*, *21*, 277–283.

Teixido, A. L., & Valladares, F. (2013). Large and abundant flowers increase indirect costs of corollas: a study of coflowering sympatric Mediterranean species of contrasting flower size. *Oecologia*, *173*, 73-81.

Temeles, E. J, & Bishop G. A. (2019). A hurricane alters pollinator relationships and natural selection on an introduced island plant. *Biotropica, 51*, 129–138.

Thompson, J. D. (2020). *Plant Evolution in the Mediterranean: Insights for conservation*. Oxford University Press, USA.

Thompson, J. N. (1998). Rapid evolution as an ecological process. *Trends in ecology & evolution*, 13, 329-332.

Thøstesen, A. M., & Olesen, J. M. (1996). Pollen removal and deposition by specialist and generalist bumblebees in *Aconitum septentrionale*. *Oikos*, 77-84.

Tiedeken, E. J., Egan, P. A., Stevenson, P. C., Wright, G. A., Brown, M. J., Power, E. F., Farrell, I., Matthews, S. M. & Stout, J. C. (2016). Nectar chemistry modulates the impact of an invasive plant on native pollinators. *Functional Ecology*, *30*, 885-893.

Traveset, A., Willson, M. F., & Sabag, C. (1998). Effect of nectar-robbing birds on fruit set of *Fuchsia magellanica* in Tierra del Fuego: a disrupted mutualism. *Functional ecology*, *12*, 459-464.

Valiente-Banuet, A., Molina-Freaner, F., Torres, A., Del Coro Arizmendi, M., & Casas, A. (2004). Geographic differentiation in the pollination system of the columnar cactus *Pachycereus pecten-aboriginum*. *American Journal of Botany, 91*, 850–855.

Valiente-Banuet, A., & Verdú, M. (2013). Human impacts on multiple ecological networks act synergistically to drive ecosystem collapse. *Frontiers in Ecology and the Environment*, *11*, 408-413.

Valladares, F., Sanchez-Gomez, D., Zavala, M. A. (2006). Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. *Journal of ecology*, *94*, 1103-1116.

Vallejo-Marín, M., & Barrett, S. C. (2009). Modification of flower architecture during early stages in the evolution of self-fertilization. *Annals of Botany*, *103*, 951-962.

Van der Niet, T., Peakall, R., & Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany*, *113*, 199-212.

van Kleunen, M., & Ritland, K. (2004). Predicting evolution of floral traits associated with mating system in a natural plant population. *Journal of Evolutionary Biology*, *17*, 1389-1399.

Varma, S., Rajesh, T. P., Manoj, K., Asha, G., Jobiraj, T., & Sinu, P. A. (2020). Nectar robbers deter legitimate pollinators by mutilating flowers. *Oikos*, *129*, 868-878.

Varma, S., & Sinu P. A. (2019). Nectar robbing in bellflower (*Sesamum radiatum*) benefited pollinators but unaffected maternal function of plant reproduction. *Scientific Reports, 9*, 1–9.

Villalona, E., Ezray, B. D., Laveaga, E., Agrawal, A. A., Ali, J. G., & Hines, H. M. (2020). The role of toxic nectar secondary compounds in driving differential bumble bee preferences for milkweed flowers. *Oecologia*, *193*, 619-630.

Vogler, A., Bertossa, M., Aulinger-Leipner, I., & Stamp, P. (2010). Weather Effects on Cross-Pollination in Maize. *Crop science*, *50*, 713-717.

Walsh, B., & Lynch, M. (2014). Theorems of natural selection: results of Price, Fisher, and Robertson. *Evolution and Selection of Quantitative Traits*.

Wang, Y., Zhang, D., Renner, S. S., & Chen, Z. (2004). A new self-pollination mechanism. *Nature*, *431*, 39-40.

Ward, M., Johnson S. D., & Zalucki, M. P. (2012). Modes of reproduction in three invasive milkweeds are consistent with Baker's Rule. *Biological Invasions, 14*, 1237–1250.

Webb, C. J., & Lloyd, D. G. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand journal of botany*, *24*, 163-178.

Whitman, D. W., & Agrawal, A. A. (2009). What is phenotypic plasticity and why is it important. Phenotypic plasticity of insects: *Mechanisms and consequences*, 1-63.

Whittall, J. B. & Hodges, S. A. (2007). Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature*, *447*, 706-709.

Williams, J. L., Auge, H., & Maron, J. L. (2008). Different gardens, different results: native and introduced populations exhibit contrasting phenotypes across common gardens. *Oecologia*, *157*, 239-248.

Willis, A. J., Memmott, J., & Forrester, R. I. (2000). Is there evidence for the post-invasion evolution of increased size among invasive plant species? *Ecology Letters, 3*, 275–283.

Wood, S. N. (2011). Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *Journal of the Royal Statistical Society (B)*, *73*, 3–36.

Worley, A. C., & Barrett, S. C. H. (2000). Evolution of floral display in *Eichhornia paniculata* (Pontederiaceae): Genetic correlations between flower size and number. *Journal of Evolutionary Biology, 14*, 469–481.

Young, H. J., Stanton, M. L., Ellstrand, N. C., & Clegg, J. M. (1994). Temporal and spatial variation in heritability and genetic correlations among floral traits in *Raphanus sativus*, wild radish. *Heredity*, *73*, 298-308.

Zimmerman, M., & Cook, S. (1985). Pollinator foraging, experimental nectar-robbing and plant fitness in Impatiens capensis. *American Midland Naturalist*, 84-91.

Zu, P., Blanckenhorn, W. U., & Schiestl, F. P. (2016). Heritability of floral volatiles and pleiotropic responses to artificial selection in *Brassica rapa. New Phytologist*, *209*, 1208-1219.

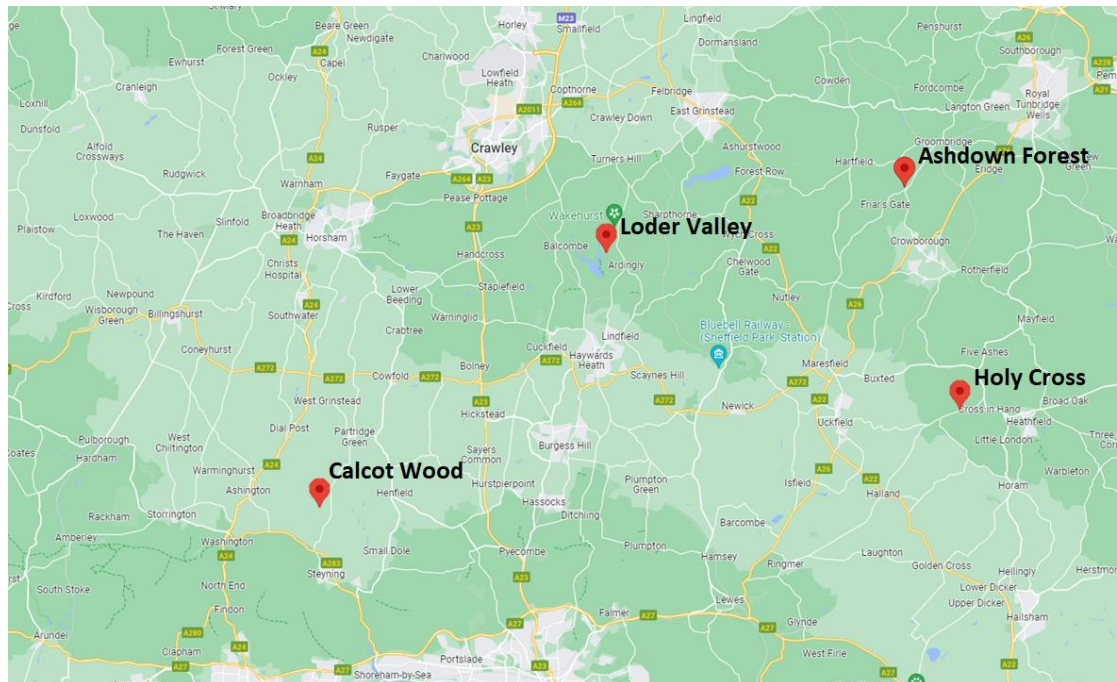# Appendices

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Appendix: Chapter 2

Figure A2.1. Map of the four *Digitalis purpurea* populations studied in Britain.

Figure A2.2. Map of the five *Digitalis purpurea* populations studied in Colombia.

Figure A2.3. Map of the two *Digitalis purpurea* populations studied in Costa Rica.
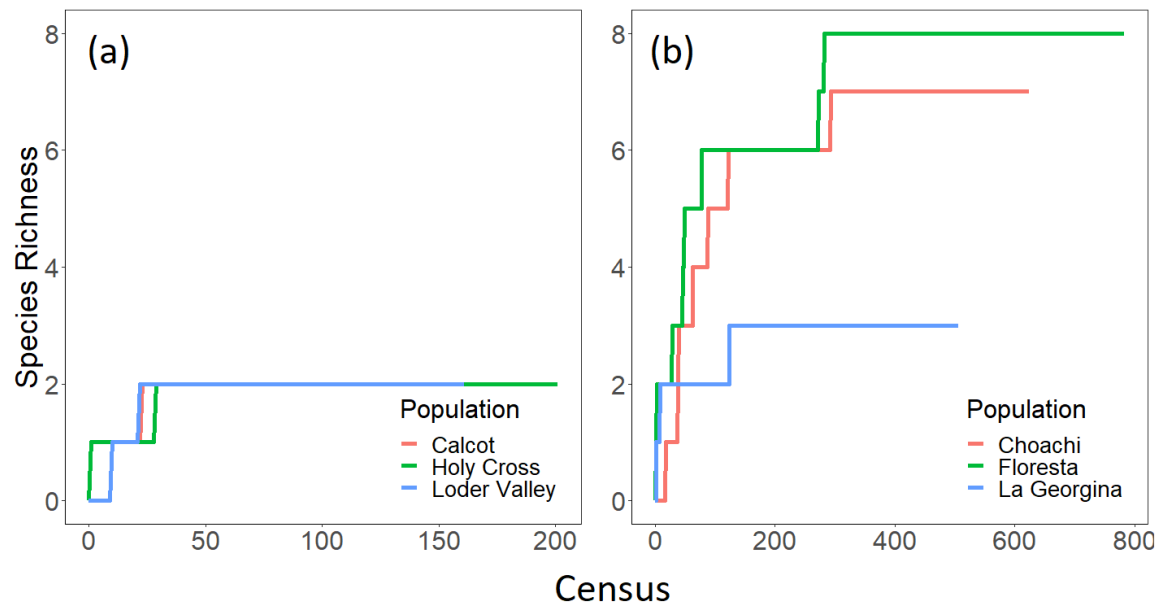
Figure A2.4. Species accumulation curves of pollinators in (a) the native range, where we performed between 140 and 200 pollinator censuses per population, and (b) the non-native range, with more than 500 censuses per population.
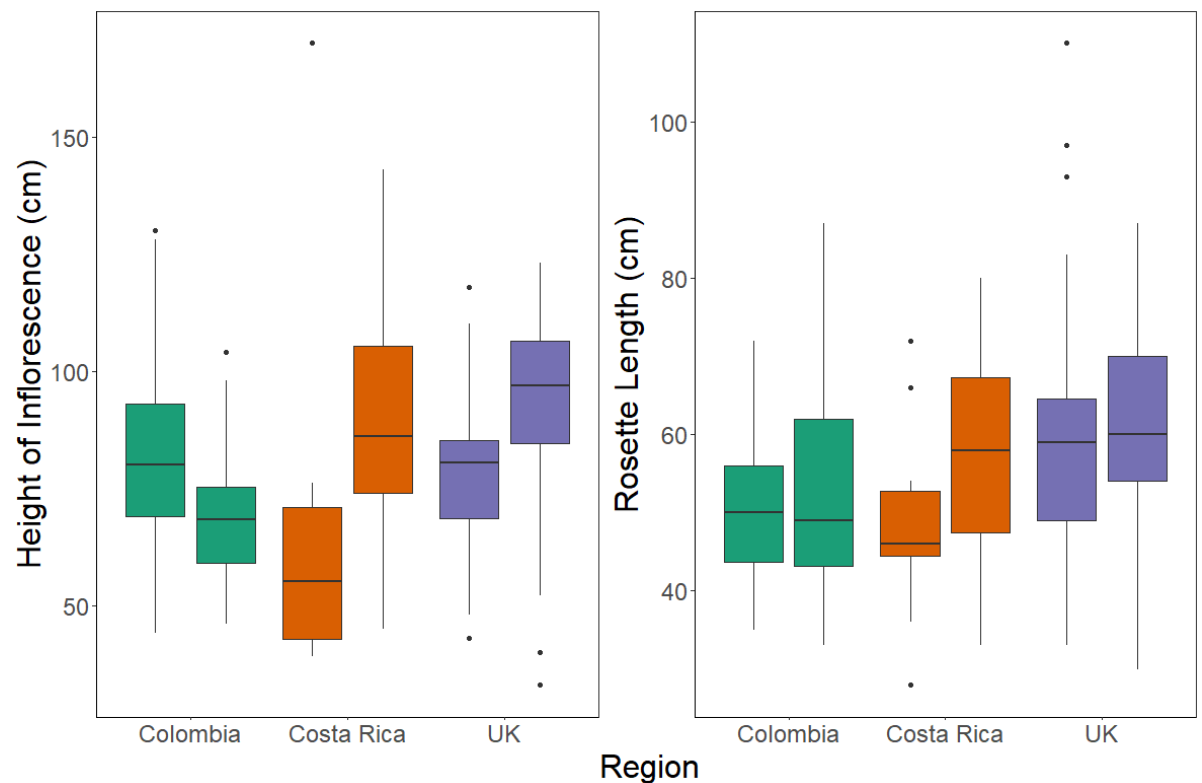
Figure A2.5. Comparison of the height of the inflorescence to the first flower (peduncle; left panel), and rosette length (= diameter; right panel) in introduced (Colombia and Costa Rica) and native (Britain) *Digitalis purpurea* populations (N = 10 to 60 plants per population).
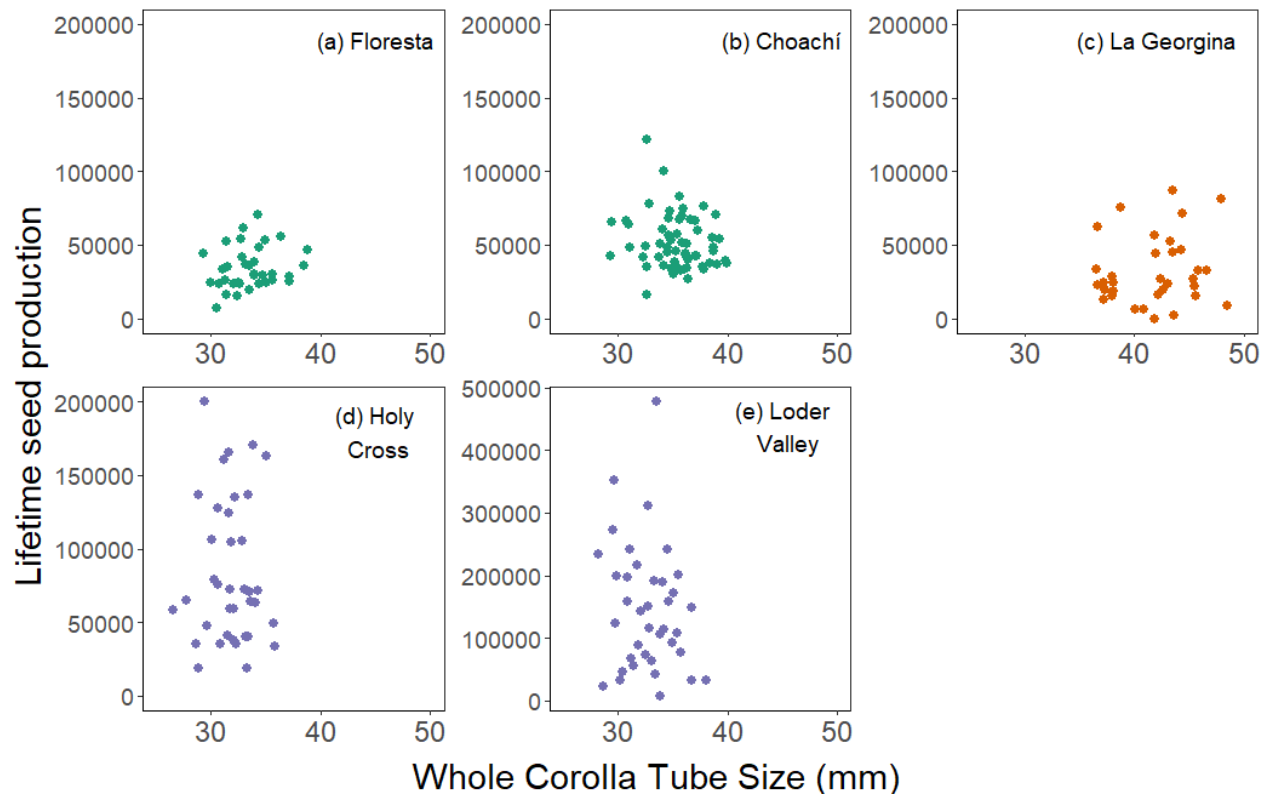
Figure A2.6. Relationship between the whole corolla tube size and the lifetime seed production in individual *Digitalis purpurea* plants. Whole corolla tube was measured as the geometric mean of proximal corolla tube length and width. Non-native populations (a) Floresta, Colombia, (b) Choachí, Colombia, and (c) La Georgina, Costa Rica. Native populations (d) Holy Cross and (e) Loder Valley in Britain.

Figure A2.7. Relationship between the proximal corolla tube size and lifetime seed production, for the native population Loder Valley in Britain. Proximal corolla tube was measured as the geometric mean of proximal corolla tube length and width.

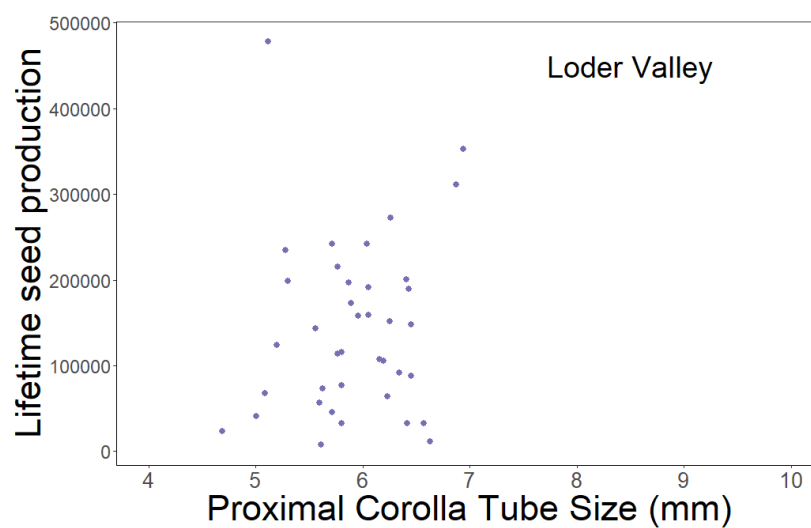**Table A2.1.** Pollinating and non-pollinating flower visitors to *Digitalis purpurea* plants in all populations. The number and percentage of visits include non-robbing visits only. Note that the surveying effort was higher in the non-native populations; number of 3-minute pollinator census per population: Loder Valley: 161 censuses, Holy Cross: 201, Calcot Wood: 140, Choachí: 624, Floresta: 524, La Georgina: 506. See main text for more details on the methods.

Region	Population	Functional Visitor Group	Species	Pollinator?	Nectar robber	Percentage of visits	Number of Visits
Britain **(native)**	Loder Valley	Bumblebee	*Bombus hortorum*	Yes		89.3	184
			Bombus pascuorum	Yes		6.3	13
		Solitary bee	*Andrena sp.*			1.0	2
		Hoverfly	*Episyrphus sp.*			3.4	7
	Holy Cross	Bumblebee	*Bombus hortorum*	Yes		83.2	407
			Bombus pascuorum	Yes		0.8	13
		Solitary bee	*Anthidium manicatum*			4.1	20
			Lasioglossum fulvicorne			1.0	5
			Lasioglossum sp.			3.9	19
			Andrena sp.			1.4	7
		Hoverfly	*Episyrphus sp.*			3.9	19
			Eumerus sp.			0.2	1
			Epistrophe sp.			0.8	4
		Honeybee	*Apis mellifera*			0.6	3
	Calcot Wood	Bumblebee	*Bombus hortorum*	Yes		94.6	501
			Bombus pascuorum	Yes		1.1	6
		Honeybee	*Apis mellifera*			1.1	6
		Solitary bee	*Lasioglossum calcarium*			0.4	5
			Lasioglossum fulvicorne			0.9	1

			Anthidium manicatum			0.9	5
		Butterfly	*Pieris brassicae*			0.2	1
			Maniola jurtina			0.8	4
Colombia (non-native)	Choachí	Bumblebee	*Bombus robustus*	Yes	Yes	60.9	414
			Bombus funebris	Yes	Yes	13.4	91
			Bombus rubicundus	Yes	Yes	4.6	31
			Bombus atratus	Yes	Yes	0.4	3
		Hummingbird	*Aglaeactis cupripennis*	Yes	Yes	1.5	10
			Eriocnemis vestita	Yes	Yes	17.5	119
			Eriocnemis cupreoventris	Yes		1.8	12
		Flower Piercer	*Diglossa sp.*		Yes		
		Honeybee	*Apis mellifera*		Yes		
	Floresta	Bumblebee	*Bombus hortulanus*	Yes	Yes	87.6	861
			Bombus atratus	Yes		6.2	61
		Hummingbird	*Coeligena bonapartei*	Yes		3.3	32
			Coeligena prunelli	Yes	Yes	0.3	3
			Heliodoxa rubinoides	Yes		0.6	6
			Coeligena torguata	Yes		1.6	16
			Chrysolampis mosquitus	Yes		0.4	4
		Honeybee	*Apis mellifera*		Yes		
		Butterfly	*Pieridae sp.*		Yes		
		Tyrant Bird	*Tyranndiae sp.*		Yes		
Costa Rica (non-native)	La Georgina	Bumblebee	*Bombus ephiphatus*	Yes	Yes	72.3	518
		Hummingbird	*Eugenes fulgens*	Yes		19	136
			Selaphorus flammula	Yes	Yes	8.7	62

Table A2.2. Bee tongue length measurements from the literature (mean ± standard deviation or standard error); N= sample size.

Region	Bumblebee Species	Tongue Length (mm)	N	Author
Native (Britain)	*Bombus hortorum*	12.9 ± 0.8 SD	10	Goulson & Darvill, 2004
	Bombus pascuorum	11.8	n/a	Kwak, 1978
		8.5 ± 0.6 SD	10	Goulson & Darvill, 2004
		8.6	n/a	Kwak, 1978
Non-native (South America)	*Bombus atratus*	8.4 ± 0.65	34	Arbulo et al., 2011
	Bombus robustus	6.9 ± 0.55 SD	5	this study
	Bombus rubicundus/hortulanus	6.9 ± 0.4 SD	10	Riveros et al., 2006
Non-native (Central America)	*Bombus ephippiatus*	11.1 ± 0.34 SE	10	Del Carmen Salas-Arcos et al., 2019

Note: Measures for *B. rubicundus and B. hortulanus* are combined in the original publication.

Table A2.3. Directional and quadratic selection coefficients (± standard errors) for the whole corolla tube length and height in each population of *D. purpurea*. Values that are statistically significant from zero are indicated by *(P < 0.05).

Trait	Country	Population	Directional (β)	Non-Linear (γ)
Whole Corolla Tube Length	Britain	Loder Valley	-0.07 ± 0.117	-0.16 ± 0.097
	Britain	Holy Cross	0.07 ± 0.099	0.00 ± 0.000
	Costa Rica	La Georgina	0.20 ± 0.123	0.00 ± 0.000
	Colombia	Floresta	0.11 ± 0.072	0.00 ± 0.000
	Colombia	Choachí	0.02 ± 0.047	0.00 ± 0.000
Whole Corolla Tube Height	Britain	Loder Valley	-0.15 ± 0.115	0.07 ± 0.072
	Britain	Holy Cross	-0.06 ± 0.101	0.00 ± 0.000
	Costa Rica	La Georgina	0.03 ± 0.129	0.00 ± 0.000
	Colombia	Floresta	0.04 ± 0.076	0.00 ± 0.000
	Colombia	Choachí	**-0.10 ± 0.044***	0.00 ± 0.000

Appendix: Chapter 3

Table A3.1. Variance Partitioning of the floral traits of *Digitalis purpurea* plants, for each population in the common garden.

Population (Range)	Level	Whole Corolla Size	Corolla Weight	Proximal Corolla Length	Proximal Corolla Width
Loder Valley (Native)	Within Individuals	5.8%	15.4%	40.8%	28.2%
	Among Plants	76.3%	70.9%	52.0%	37.3%
	Among Families	18.0%	13.7%	7.2%	34.5%
Holy Cross (Native)	Within Individuals	19.1%	24.0%	35.8%	39.5%
	Among Plants	43.3%	60.8%	24.3%	33.9%
	Among Families	37.5%	15.3%	39.9%	26.6%
Floresta (Introduced)	Within Individuals	15.9%	23.7%	36.7%	56.2%
	Among Plants	84.1%	76.3%	63.3%	29.1%
	Among Families	0.0%	0.0%	0.0%	14.6%
La Georgina (Introduced)	Within Individuals	18.6%	23.2%	41.0%	60.2%
	Among Plants	81.4%	54.5%	43.3%	38.1%
	Among Families	0.0%	22.2%	15.7%	1.7%

Table A3.2. Significant heritabilities of corolla traits of various species from published studies. The criteria for inclusion are heritability estimates using approaches comparable to our own that find estimates with significant difference from zero. The approaches include parent offspring (PO) regression, paternal half sibs and diallel. Maternal (dam), paternal (sire) and mid (mid) parent offspring regressions are included. Standard error (SE) for each estimate from each study is included when given. The papers were found from Table S3 in Opedal, 2019.

Trait	Author	Species	Method	h^2	SE
Corolla diameter	Elle, 1998	*Lobelia siphilitica*	Diallel	0.31	NA
	Elle, 1998	*Lobelia siphilitica*	Diallel	0.37	NA
	Elle, 1998	*Lobelia siphilitica*	Diallel	0.45	NA
Corolla length	Campbell, 1996	*Ipomopsis aggregate*	PO regression (sire)	0.74	0.22
	Carr & Fenster, 1994	*Mimulus guttatus*	PO regression (dam)	0.523	0.398
	Carr & Fenster, 1994	*Mimulus guttatus*	PO regression (mid)	0.576	0.330
	Galen & Cuba, 2001	*Polemonium viscosum*	PO regression (dam)	0.74	0.29
	Van Kleunen & Ritland, 2004	*Mimulus guttatus*	PO regression (dam)	0.213	0.068
	Young et al., 1994	*Raphanus sativus*	PO regression (mid)	0.41	0.076
	Schwaegerie & Levin, 1991	*Phlox drummondii*	Paternal half sibs	0.14	NA
	Bissell et al., 2010	*Nicotiana alata*	Paternal half sibs	0.720	NA
	Bissell et al., 2010	*Nicotiana forgetiana*	Paternal half sibs	0.767	NA
	Mitchell & Shaw, 1993	*Penstemon centranthifoliu*	Half sibs	0.68	NA
	Caruso, 2004	*Lobelia siphilitica*	Half sibs	0.663	NA
	Caruso, 2004	*Lobelia siphilitica*	Half sibs	0.565	NA
	Campbell, 1996	*Ipomopsis aggregate*	Nested ANOVA	0.24	NA
	Kaczorowski et al., 2008	*Nicotiana alata*	Diallel	0.17	NA

|	Reference	Species	Method		
	Ritland & Ritland, 1996	*Mimulus guttatus*	PO regression (dam)	0.34	0.16
	Ritland & Ritland, 1996	*Mimulus guttatus*	PO regression (dam)	0.42	0.15
	Carr & Fenster, 1994	*Mimulus guttatus*	PO regression (dam)	0.296	0.266
	Carr & Fenster, 1994	*Mimulus guttatus*	PO regression (mid)	0.376	0.330
	Fenster & Carr, 1997	*Mimulus guttatus*	PO regression (dam)	0.296	0.132
	Fenster & Carr, 1997	*Mimulus guttatus*	PO regression (dam)	0.313	0.184
	Fenster & Carr, 1997	*Mimulus guttatus*	PO regression (mid)	0.376	0.165
Corolla width	Fenster & Carr, 1997	*Mimulus guttatus*	PO regression (mid)	0.157	0.138
	Ritland & Ritland, 1996	*Mimulus guttatus*	PO regression (dam)	0.34	0.16
	Ritland & Ritland, 1996	*Mimulus guttatus*	PO regression (dam)	0.42	0.15
	Bissell et al., 2010	*Nicotiana alata*	Paternal half sibs	0.282	NA
	Bissell et al., 2010	*Nicotiana forgetiana*	Paternal half sibs	0.319	NA
	Caruso, 2004	*Lobelia siphilitica*	Half sibs	0.655	NA
	Caruso, 2004	*Lobelia siphilitica*	Half sibs	0.631	NA
	Mitchell & Shaw, 1993	*Penstemon centranthifoliu*	Half sibs	0.48	NA
	Campbell, 1996	*Ipomopsis aggregate*	Nested ANOVA	0.29	NA

Appendix: Chapter 4
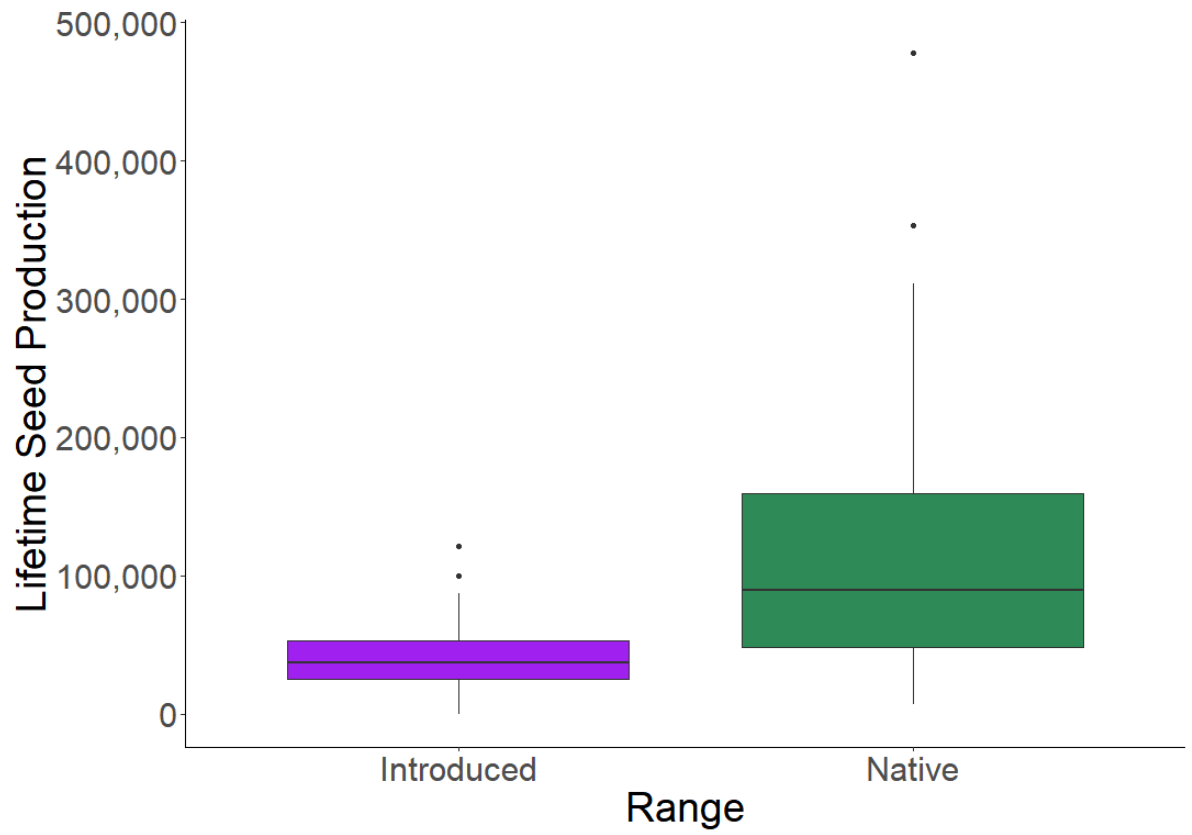
Figure A4.1. Total number of seeds produced in plants across three non-native populations in Colombia and Costa Rica (purple, N = 135 plants) and two native populations in Britain (green, N = 76).
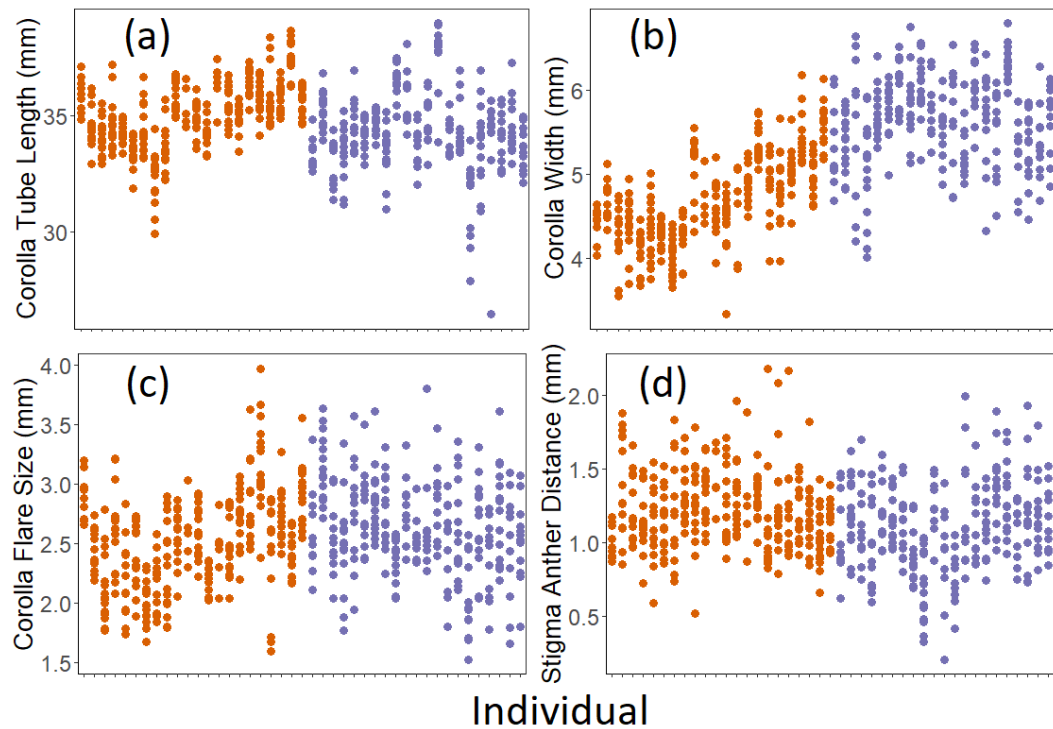
Appendix: Chapter 5

Figure A5.1. Distribution of variation among plants in 4 traits in Chayofa (N=303, orange) and El Rio (N=271, purple) populations, including (a) Corolla Tube Length; (b) corolla tube width, (c) corolla flare size and (d) stigma anther distance.
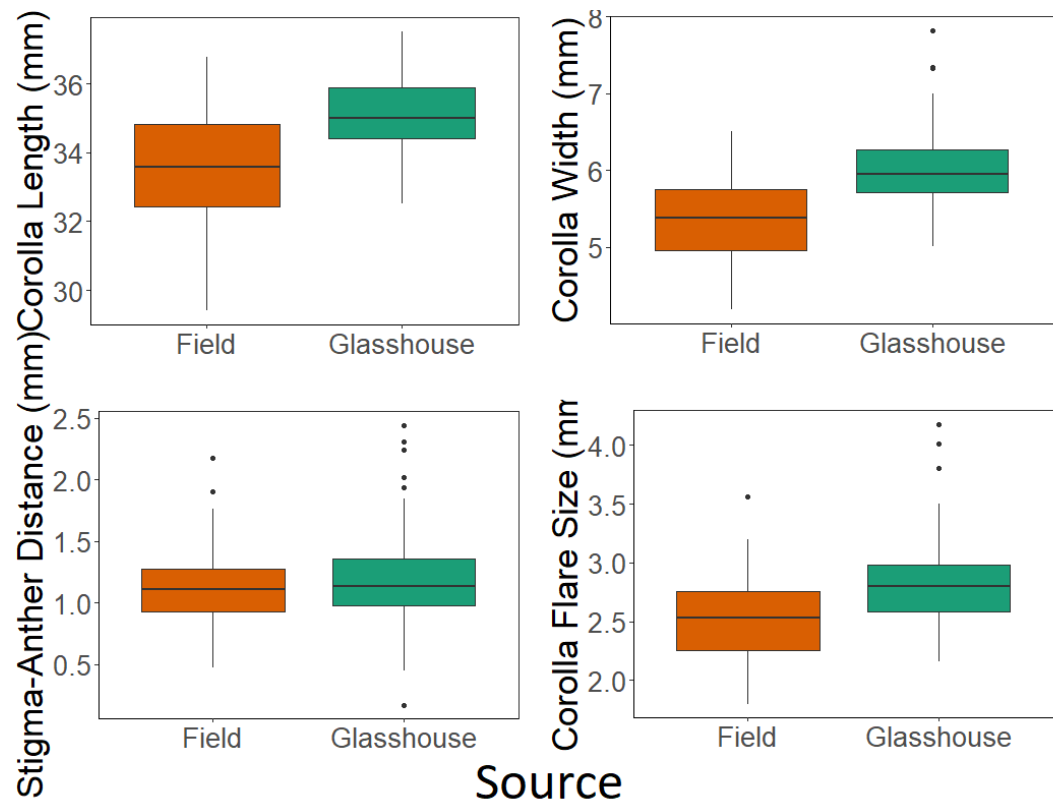

Figure A5.2. A comparison of the Chayofa field population (N=84) with the Chayofa common garden plants (N=90) for Corolla Length, Corolla Width, SA Distance and Corolla Flare Size.
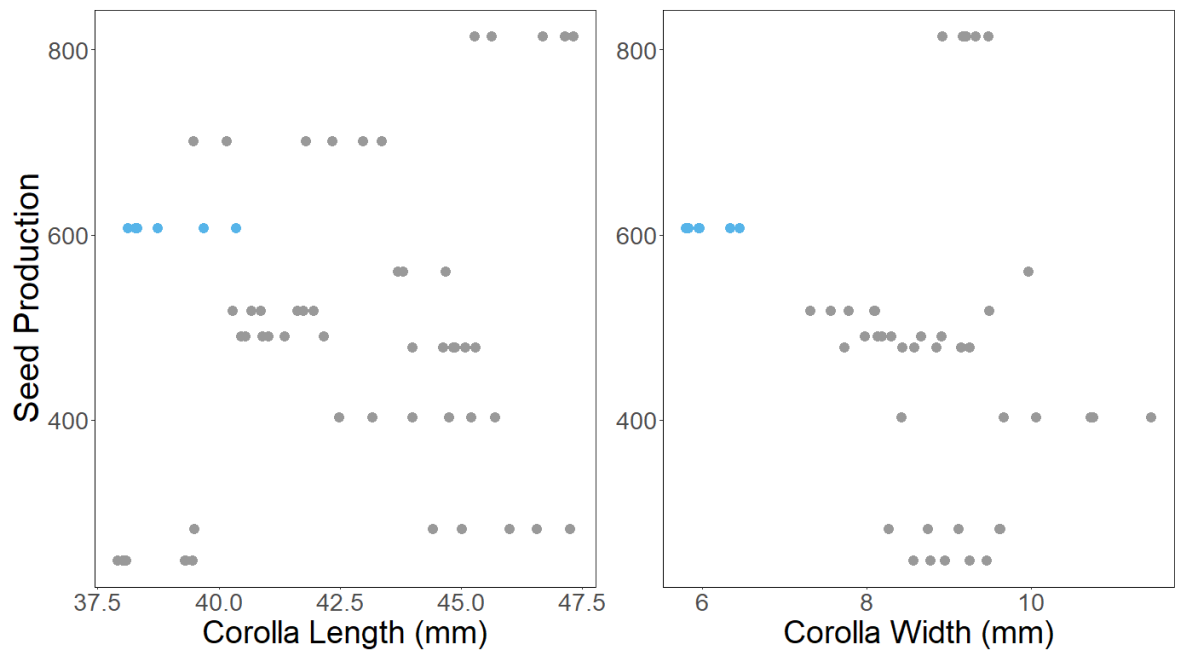
Figure A5.3. Relationship between corolla length (N = 56) and corolla width (N = 46) with mean seed production of flowers in the common garden plants. Plants are sourced from mothers in Tupiza in Argentina (grey) and Buffel's River in South Africa (blue).
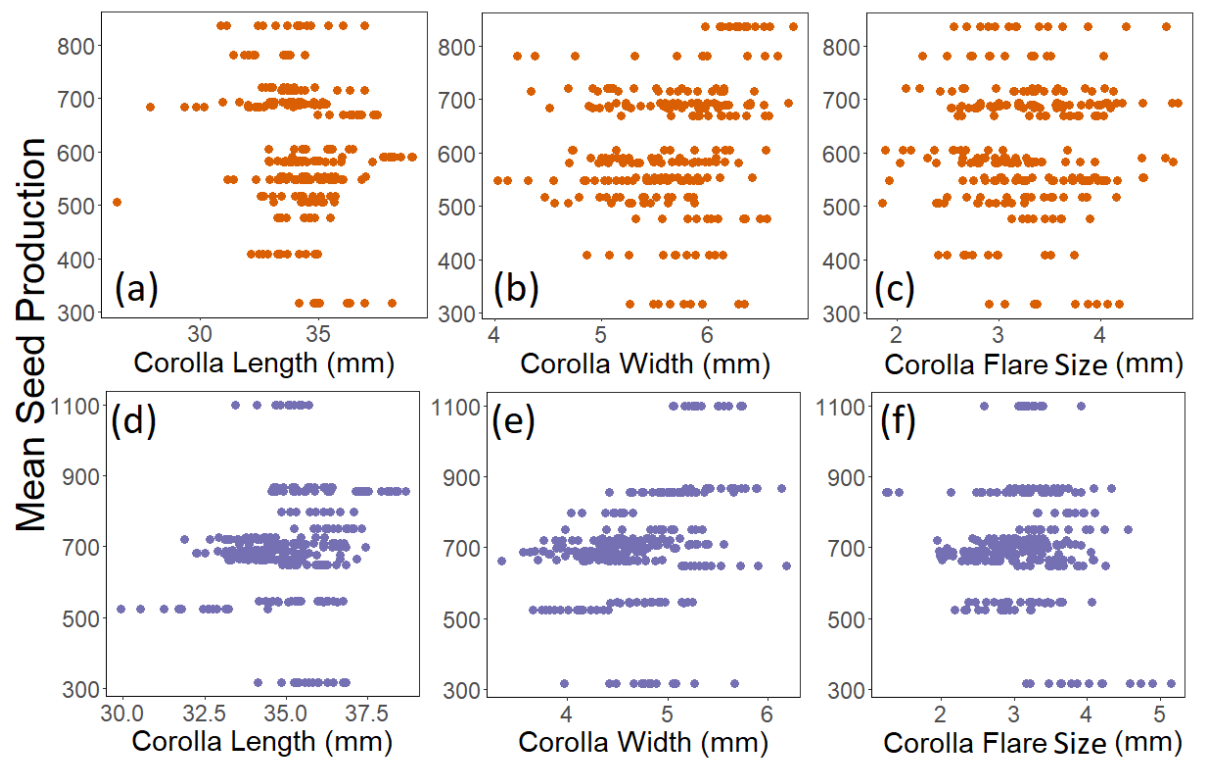

Figure A5.4. Relationship between floral traits and the mean seed production of flowers in Chayofa (N = 303) (a-c) and El Rio (N = 271) (d-f).
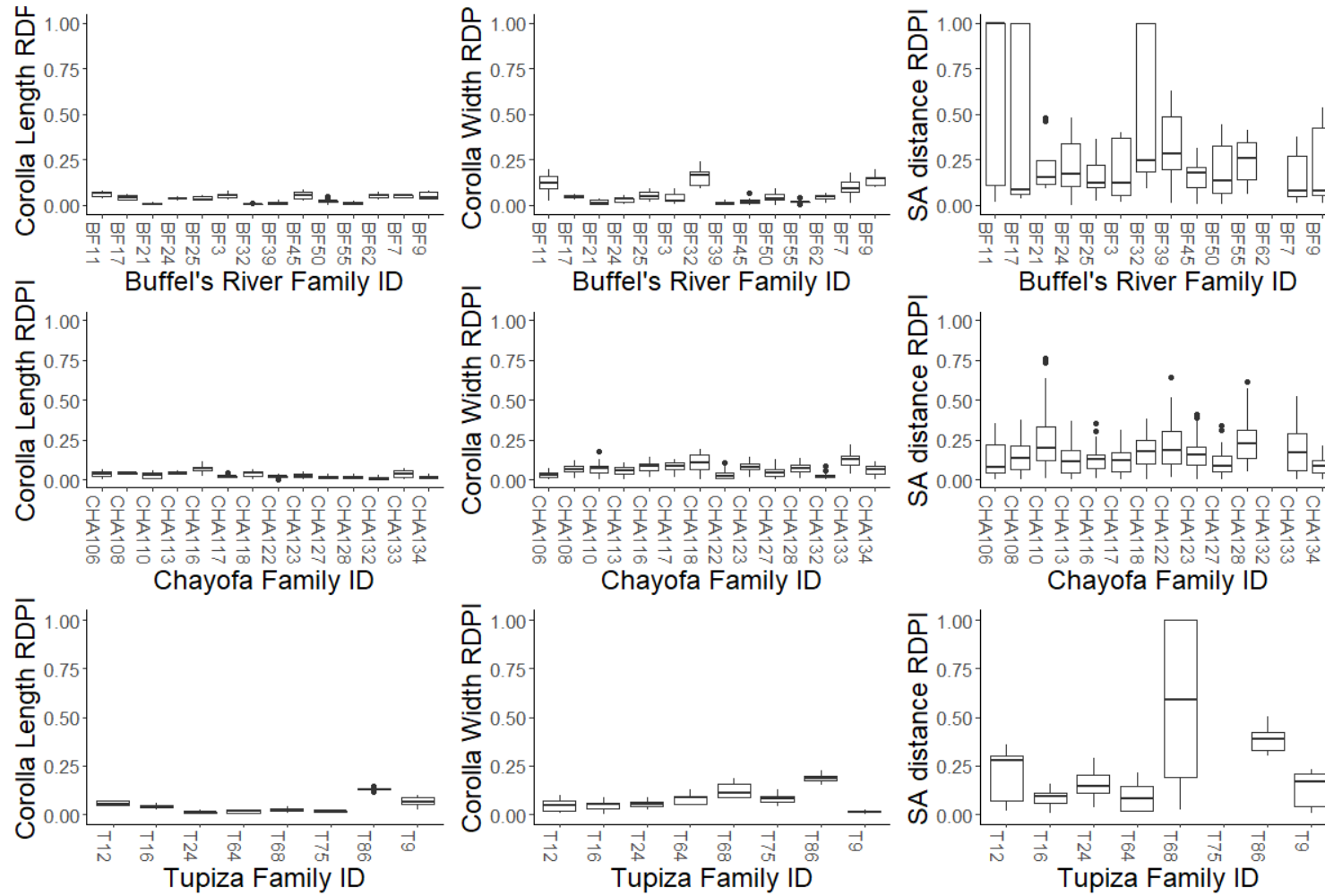
Appendix: Chapter 6

Figure A6.1. Relative distance plasticity index (RDPI) for corolla length, corolla width and SA distance in Buffel's River (N = 84 flowers from 28 plants), Chayofa (N = 169 flowers from 28 plants) and Tupiza (N = 48 flowers from 16 plants).

Table A6.1. Significant heritabilities of herkogamy for various species from published studies, focussing on methods comparable to our own, such as parent-offspring regressions and diallels. Error around the estimate is included when given, shown as standard error (SE) or confidence interval (CI). Data collected from papers cited in Table S3 in Opedal, 2019. Estimates from the same publication are for separate populations or groups.

Method	Author	Species	h^2	Error
Uniparental regression	Carr & Fenster, 1994	*Mimulus guttatus*	0.345	0.340 (CI)
	Carr & Fenster, 1994	*Mimulus guttatus*	0.763	0.302 (CI)
	Van Kleunen & Ritland, 2004	*Mimulus guttatus*	0.280	0.105 (SE)
Midparent-offspring regression	Carr & Fenster, 1994	*Mimulus guttatus*	0.378	0.288 (CI)
	Carr & Fenster, 1994	*Mimulus guttatus*	0.557	0.270 (CI)
Family variance	Fishman et al., 2002	*Mimulus sp.*	0.34	NA
	Herlihy & Eckert, 2007	*Aquilegia canadensis*	0.78	0.36 – 1.19 (CI)
	Herlihy & Eckert, 2007	*Aquilegia canadensis*	0.38	0.15 – 0.61 (CI)
	Herlihy & Eckert, 2007	*Aquilegia canadensis*	0.21	-0.01 – 0.42 (CI)
	Herlihy & Eckert, 2007	*Aquilegia canadensis*	0.54	0.05 – 1.03 (CI)
	Herlihy & Eckert, 2007	*Aquilegia canadensis*	0.20	-0.06 – 0.46 (CI)
	Shore & Barrett, 1990	*Turnera ulmifolia*	0.572	NA
Diallel	Hansen et al., 2003	*Dalechampia scandens*	0.26	NA
	Motten & Stone, 2000	*Datura stramonium*	0.20	NA
	Motten & Stone, 2000	*Datura stramonium*	0.31	NA
	Motten & Stone, 2000	*Datura stramonium*	0.30	NA
Pedigree	Kulbaba & Worley, 2008	*Polemonium brandegei*	0.851	NA

Table A6.2. Variance Partitioning of *Nicotiana glauca* floral traits, for each population in the common garden.

Population (Range)	Level	Corolla Length	Corolla Width	SA Distance
Tupiza (Native)	Within Individual	38.8%	16.5%	70.7%
	Between Plants	40.9%	21.1%	26.5%
	Between Families	20.4%	62.3%	2.8%
Piedra Pintada (Native)	Within Individual	15.6%	38.7%	26.8%
	Between Plants	29.7%	61.3%	59.0%
	Between Families	54.6%	0.0%	14.2%
Buffel's River (Introduced)	Within Individual	44.0%	43.2%	81.3%
	Between Plants	42.5%	41.4%	13.5%
	Between Families	13.5%	15.4%	5.2%
Génova (Introduced)	Within Individual	63.4%	63.2%	89.2%
	Between Plants	25.4%	36.8%	10.8%
	Between Families	11.1%	0.0%	0.0%
Chayofa (Introduced)	Within Individual	40.5%	49.8%	85.6%
	Between Plants	45.3%	29.6%	9.2%
	Between Families	14.2%	20.6%	5.2%