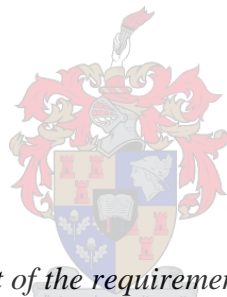


The convergence and divergence of floral traits are driven by the heterogeneity of pollinator and plant communities



Thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy in the

Faculty of Science at Stellenbosch University

By

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Declaration

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Abstract

Substantial evidence suggests that pollinators are responsible for generating floral variation when they select upon floral traits involved in attraction as well the efficiency of pollen transfer. Another source of floral variation that is often neglected, is the role of community structure which drives floral divergence of traits involved in competition for the same pollinator resource. When these traits are selected upon across a heterogeneous landscape, it is thought to give rise to the formation of local floral forms which are often referred to as ecotypes.

This thesis uses the long-proboscid fly (*Prosoeca longipennis*) study system as a platform for asking questions regarding the generation of floral variation. In my first data chapter (chapter two), I show how this variation can arise when tube length converges upon fly proboscis length across several localities, leading to a pattern of geographic trait matching. In addition, I also identify populations of species which appear to be morphologically divergent, because in populations where *P. longipennis* is absent, they are often pollinated by morphologically different pollinators. In chapter three, I investigate pollinator driven floral divergence in *Nerine humilis*, a species pollinated by long-proboscid flies in some populations and short proboscid insects in others. In this chapter, I demonstrate local adaptation of different floral forms associated with different pollinators. In addition, I take an extra step and demonstrate that the mechanical fit between flower and pollinator morphology is the mechanism behind local pollinator adaptation. In my fourth chapter, I show that floral adaptation may not always be as clearly illustrated as in chapter three, because most plants are visited by a multitude of functionally different pollinators. Here, I explore pollinator-mediated selection in *Tritoniopsis revoluta* and *Nerine humilis* with multiple functional pollinator types. Using single visitations, I demonstrate that flowers adapt

to the optima and slopes of the additive fitness functions from all functional pollinator types. In my last data chapter, chapter five, I demonstrate how floral divergence may occur through the context of the floral guild community, where floral divergence does not occur through selection exerted by pollinators, but occurs as a result of competition for the placement of pollen on the bodies of long-proboscid fly's across different localities. This resultant process of ecological character displacement gives rise to a pattern where mean style lengths of *Pelargonium*s are more different when they co-occur compared to when they occur on their own, where they may have style lengths that are similar or different to those in sympatry.

This thesis contributes to the existing literature by providing much needed evidence on how selection exerted by pollinators as well as the structure of the floral guild community may drive adaptive divergence of floral morphology across a heterogeneous landscape.

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“To a person uninstructed in natural history, his country or sea-side stroll is a walk through a gallery filled with wonderful works of art, nine-tenths of which have their faces turned to the wall. Teach him something of natural history, and you place in his hands a catalogue of those which are worth turning around”
– Thomas Huxley.

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Chapter 1

INTRODUCTION

The role of pollinators in driving angiosperm diversification has always been mired in controversy: Some authors provide evidence that suggests a pivotal role played by the coevolution of angiosperms and pollinating insects (Dodd *et al.*, 1999; Grimaldi, 1999), while others argue that insects appeared before angiosperms and show no apparent diversification during the angiosperm radiation (Labandeira & Sepkoski, 1993). Nonetheless, mounting evidence clearly suggests that floral variation has been shaped by pollinator-mediated selection at the microevolutionary scale (Worley & Barret, 2000; Alexandersson & Johnson, 2002; Benitez - Vieyra *et al.*, 2006; Sletvold & Agren, 2010; Vamosi & Vamosi, 2010). Pollinator-mediated selection, either through the mechanical or sensory fit between flower and pollinator morphology may lead to floral divergence; especially when plants traverse a heterogeneous pollinator landscape (Grant, 1949; Grant & Grant, 1965). This process may eventually lead to the formation of pollination ecotypes, local floral forms that have diverged as a result of differences in functional pollinator type (Robertson & Wyatt, 1990).

Floral variation can also evolve in response to pollinators without the pollinator species composition varying geographically (*see* Ellis and Anderson, 2012 for review). Here, pollinators of the same species may vary geographically in terms of their morphology and/or behavior. For example, reciprocal selection between pollinator (insect proboscis length) and flower morphology (corolla depth) across different populations may lead to coevolutionary races between insect and plant morphology in different populations (Thompson, 2005). Heterogeneity in the abiotic

environment is likely to stop those coevolutionary races at different endpoints in different populations, leading to geographic variability in both plant and pollinator traits (Steiner & Whitehead, 1990b; Anderson & Johnson, 2008; Pauw *et al.*, 2009; Paudel *et al.*, 2016). Floral divergence can also occur through variation in the behavior of pollinators across a geographic mosaic. For example, Swiss and Italian populations of *Colletes cunicularius* display variation in their attraction to floral extracts of *Ophrys* orchids (Mant *et al.*, 2005).

Another driver of floral variation, for which there is little evidence (Beans, 2014; Van der Niet *et al.*, 2014a), is the composition of floral community structure. An example by Newman *et al.* (2012) demonstrates that the rewardless orchid *Disa ferruginea* deceptively gains pollination services by tracking the color of rewarding plants visited by the butterfly *Aeropetes tulbaghia*. These rewarding plants differ geographically in terms of species and colour, leading to geographically variable colour preferences by *A. tulbaghia*. However, a more common and possibly underrepresented mechanism of floral divergence can occur through character displacement. Character displacement occurs within communities where organisms compete for the same resource, leading to divergent resource use in sympatry versus allopatry. This generates a pattern where species are more different in sympatry versus allopatry. The textbook example includes two species of Darwin's finch which can have similar beak depths in allopatry, but which evolve differences in beak size to reduce competition in sympatry (Lack, 1947; Brown & Wilson, 1956).

Floral divergence through character displacement may occur if species reduce interspecific pollen transfer by placing pollen on different parts of the pollinators body or by diverging in attractive traits to evoke assortative mating (e.g. floral constancy) (Muchhala & Potts, 2007; Norton *et al.*, 2015). For example, if the mixing

of pollen on the body of a pollinator has negative consequences for male or female fitness, plants may evolve mechanisms to prevent pollen mixing when they occur in sympatry. As a result, we may expect floral traits to be more similar in allopatry than they are in sympatry. Character displacement in flowering plants can take two forms: It can occur either through ecological character displacement (ECD) where community members have already completed reproductive isolation (Armbruster *et al.*, 1994; Muchhala & Potts, 2007). Alternatively it can occur through reproductive character displacement (RCD) (Hopkins & Rausher, 2012), where plants diverge in floral morphology as a result of selecting against the formation of unfit hybrids. This is synonymous with the idea of reinforcement which helps to sharpen species boundaries through reproductive isolation in secondary contact zones (Scott Armbruster & Muchhala, 2009; Hopkins, 2013).

South Africa is well known for its overwhelming floral diversity and only recently have we started to explore the processes underlying floral diversification (van der Niet *et al.*, 2014a). In particular, no South African study has investigated floral divergence through character displacement, despite evidence to suggest that it may occur in long-proboscid fly pollination systems (Goldblatt & Manning, 2000b). Goldblatt and Manning (2000a) found that different plant species belonging to the *Moegistorhynchus longirostris* guild placed pollen on different parts of the long-proboscid flies' body when they occurred in sympatry.

While character displacement has the potential to drive floral divergence, it is well documented that geographic differences in pollinator fauna frequently drive floral divergence in South African systems (reviewed by van der Niet *et al.* 2014). In particular, the ranges of long-proboscid flies tend to be patchy and may only encompass small parts of plant species ranges. Consequently, those plants are

frequently visited by different suites of pollinators in different parts of their range (Newman *et al.*, 2014; Anderson *et al.* 2014). Thus, long-proboscid fly pollination systems provide the potential for floral variation to evolve through selection driven by geographic differences in floral communities as well as pollinators.

The *Prosoeca longipennis* pollination system.

The Greater Cape Floristic Region of Southern Africa (GCFR) has earned its reputation as being one of the most florally diverse regions of the world (Goldblatt & Manning, 2002). Although strong evidence suggests that variability in the abiotic environment is the most important driver of speciation in the Cape (Van der Niet & Johnson, 2009; Verboom *et al.*, 2015), the GCFR has a particularly high diversity of specialized pollination systems, which may likely act in concert with habitat shifts to explain the exceptionally high species richness of the Cape (Anderson, *et al* 2014; Ellis and Anderson, 2012). These specialized pollination systems include oil collecting bees (Pauw, 2006), Table Mountain Pride butterflies (Johnson, 1994), nectarivorous birds (Geerts, 2011), as well as the enigmatic long-proboscid flies (Goldblatt & Manning, 2000b).

The long-proboscid fly systems of South Africa illustrate how variable these pollination guilds can be in terms of morphology (e.g. proboscis length) which highlights their importance as potential drivers of floral variation in the Cape (Fig.1). Long-proboscid fly pollination or “rhinomyophily” is the pollination by Nemestrinid or Tabanid flies with proboscides exceeding 15mm in length (Goldblatt and Manning 2000a). Long-proboscid fly pollination was first recorded in South Africa by Marloth (1908) and described in more detail at a later stage by Vogel (1954). In their synthesis publication “The long-proboscid fly pollination system in Southern Africa”. Goldblatt

and Manning (2000a) recorded seven species of long-proboscid fly and defined three principal pollination guilds based on the zoogeography and phenophase of the flies. This variation in zoogeography and phenophase of different fly species may generate a so called geographical pollinator mosaic, which may act as a “factory” for generating floral variation and potentially driving speciation events in South Africa (Johnson, 2006).

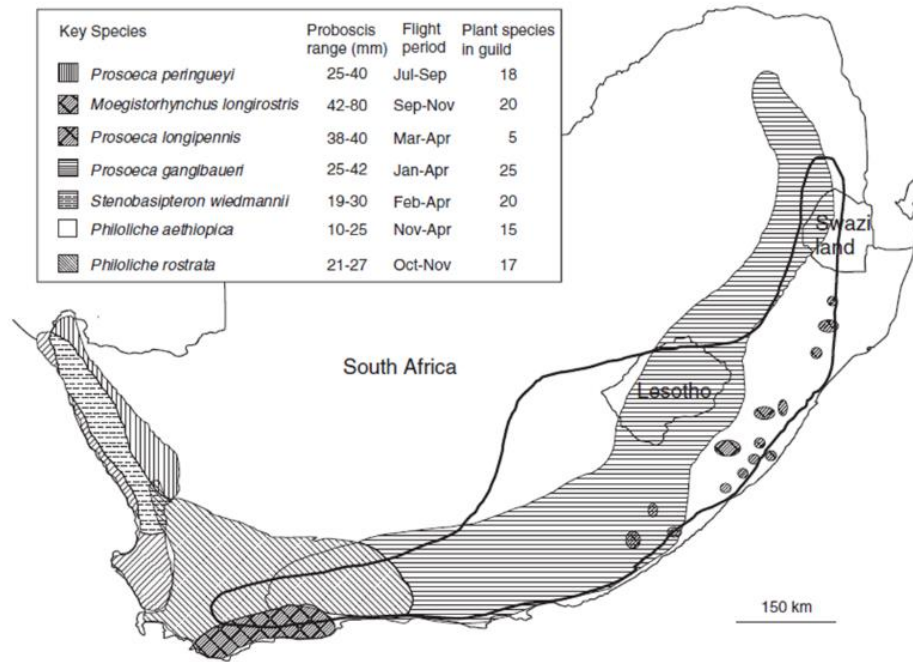


Figure. 1.1: A map of South Africa indicating the distributions of seven long-proboscid fly species. The text box provides a key to the fly guilds plotted on the map. Information on the variation in proboscis length, phenophase and the number of plant species in each pollination guild is provided. From Johnson (2006), *Ecology and Evolution of Flowers*. Edited by Harder L. and Barret, S.C.H. © 2006 Oxford University Press.

Prior to this thesis, the *Prosoeca longipennis* guild was the most poorly documented of the long-proboscid fly pollinator guilds depicted in Fig. 1.1. *Prosoeca longipennis* is a large species of long-proboscid fly characterized by bright orange hairs on the side of its thorax and abdomen (Fig.2). The species was described in 1858 by the Dutch entomologist H. Loew, and according to the key of South African Nemestrinidae (1924), *P. longipennis* was captured several times in South Africa. However, only one female existed in collections from Willowmore, in the Eastern Cape of South Africa and only after 70 years was the fly seen again at the farm Bosfontein near Riversdale in the Southern Cape. At the time of the rediscovery, five pollination guild members were described from two localities in a short popular publication “Veld and Flora” from the Botanical Society of South Africa (Manning & Goldblatt, 1995). Subsequent research, mostly as a result of this thesis, now suggests that *P. longipennis* is an important keystone pollinator of more than 17 plant species. These guild members tend to have long tubes or floral nectaries and flower in Autumn in the Southern and Eastern Cape, as well the Grassland biome of South Africa (Newman *et al.*, 2014). At many sites, the closely related *P. ganglebauri* co-occur and although their flight times slightly overlap, *P. longipennis* emerges towards the end of the *P. ganglebauri* phenophase. Of the 17 plant species now known to be visited by *P. longipennis*, several appear to have ranges that extend beyond that of *P. longipennis* where they appear to have different floral features. In addition to this, there is also considerable intraspecific floral variation within the range of *P. longipennis*.



Figure. 1.2. The long-proboscid fly *Prosoeca longipennis* approaching a flower of *Pelargonium dipetalum* at one of the first recorded localities of the fly at Suurbraak in the Western Cape of South Africa (*see* chapter three).



Figure. 1.3. A photograph of the ventral surface of a *P. longipennis* specimen from Bosfontein, near Riversdal in the Western Cape of South Africa. The photograph indicates a graticule calibrated to measure the length of the body of the fly from the base of the proboscis to the tip of the abdomen in 2mm increments. Notice the light yellow pollen from *Pelargonium pinnatum* on the “chin” of the fly which is smaller in size compared to the larger orange pollen grains from *Pelargonium carneum* on the fly’s thorax.

Research objectives

This thesis aims to address the processes that give rise to floral variation within the broader *P. longipennis* pollination system. I have structured the thesis to identify, first how floral divergence occurs through pollinator shifts followed by how floral divergence occurs through plant community structure. The first data chapter (chapter two) describes the *P. longipennis* pollination guild and identifies guild members that are pollinated by different species or suites of functional pollinator types (i.e. generalists) outside of the fly's range. More importantly, the second chapter demonstrates how floral variation can evolve through geographic trait matching between fly proboscis length and floral tube length. From here, each of the subsequent chapters focuses in on a different part of the floral guild described in chapter two. The third chapter focuses on intraspecific floral variation in the Amaryllid *Nerine humilis*. This chapter examines local adaptation in *N. humilis* and the mechanisms underlying local adaptation. The fourth chapter investigates pollinator-mediated selection on mechanical fit, conducted at several populations of the Irid *Tritoniopsis revoluta* (Iridaceae) and *Nerine humilis* (Amaryllidaceae) which contains more than one functional pollinator type. This chapter aims to tease apart selection by individual functional pollinator types within populations as well as geographic variations in the selection surfaces imposed by different pollinator communities. The fifth and final chapter investigates how geographic variation in plant community structure drives floral divergence. This chapter is different from all the previous chapters because we examine a case where floral divergence is not driven by pollinator variation. Instead, we investigate how interspecific pollen transfer can lead to niche divergence in sympatry. Here, the niche being investigated is the body of the pollinator and we hypothesize that the negative consequences of interspecific pollen transfer will drive

sympatric divergence in the reproductive morphology of competing species (character displacement). Below I state the research objectives of each data chapter in more detail.

Chapter 2: Natural selection on traits that enhance the mechanical or sensory fit between flowers and pollinators may lead to the formation of pollination syndromes. Syndromes form when unrelated plants converge upon a similar suite of floral traits to gain more efficient pollination services from a similar pollinator or functional pollinator group (Fenster *et al.*, 2004). When pollinators select upon floral traits that enhance the mechanical and sensory fit between flower and pollinator morphology across a geographic landscape, one expects floral variation should arise through the matching of floral and pollinator morphology across populations (Newman *et al.*, 2014). This matching should lead to the convergence and divergence of floral traits and may result in pollinator ecotype formation (Van der Niet *et al.*, 2014a). In this chapter, we first describe the *P. longipennis* pollination guild and identify extra-guild members pollinated by different functional pollinator types outside the range of the flies'. We predict that 1.) fly proboscis length and floral tube length will be closely matched as a result of local pollinator morphology, which should manifest itself 2.) as correlated patterns of geographic trait covariation between plant and pollinator morphology.

Chapter 3: Geographic variation in floral morphology is often assumed to reflect geographic variation in pollinator communities and associated divergence in selective pressures (Newman *et al.*, 2015). Patterns of association between flower and pollinator morphology are often inferred to represent locally adapted forms which

may lead to the formation of pollination ecotypes (Van der Niet *et al.*, 2014a). However, correlative studies are not definitive evidence for pollination ecotype formation as they do not distinguish between cause and effect (Nuismer *et al.*, 2010). Consequently, researchers have recently started to test for local adaptation using reciprocal translocations or selection experiments, but very often these studies do not investigate the actual mechanisms behind local adaptation. In particular, they do not investigate whether local adaptation mirrors the mechanical fit or sensory fit between flower and pollinator morphology. We studied *Nerine humilis* (Amaryllidaceae) across its range in 11 populations which displays a large amount of variation in style length (27.44-43.14mm). We predict that if pollinator mosaics have generated floral divergence, then populations with similar visitor assemblages will share similar floral morphology and that the styles within each population will match the functional body length of visitors. Secondly, if this is the case, we predict that local phenotypes should perform better than morphologically different introduced phenotypes in reciprocal translocations. Finally, if our predictions are met, we expect local forms to be favored over introduced forms through the mechanical or sensory fit between flowers and pollinator phenotype.

Chapter 4: Mounting evidence suggests that pollinator-mediated selection is an important contributor to the radiation of angiosperms (Harder & Johnson, 2009). However radiations in response to pollinators require that geographically variable pollinator communities impose geographically variable selection mosaics on plants (Johnson, 2006; Vamosi & Vamosi, 2010). Yet, very few studies have investigated pollinator-mediated selection across geographic pollinator landscapes (Chapurlat *et al.*, 2015). Plant populations specialized on single functional pollinators are expected

to experience strong divergent selection which may generate floral variation (Armbruster, 2014). However, the form of selection on plants with generalist pollination systems visited by multiple functional pollinator types are less certain because pollination by different functional pollinator types may lead to conflicting selection which may result in complex fitness surfaces with multiple peaks and valleys (Sahli & Conner, 2011). To break down pollinator driven selection on generalist flowers into its multiple pollinator components, one can examine how different floral traits affect pollen transfer efficiency after single visits from different pollinators. This allows one to determine selection gradients from each functional pollinator type within each population. In chapter four, we tested the following hypotheses: 1) we predict that functionally different pollinators may exert contrasting patterns of selection and that 2) morphologically similar pollinators are likely to exert similar selective pressures on plant populations which share pollinators. 3) We predict that populations with functionally different suites of pollinators should experience divergent selection and 4) if the mechanical fit between flower and pollinator morphology is responsible for the shape of the fitness surface, we expect to find matches between the fitness peaks in different populations and the morphology of the most important pollinators. We also expect that the average floral traits in each population should also correspond to calculated fitness peak

Chapter 5: Competition leading to ecological character displacement or ECD (divergent forms of a species as a result of resource competition between reproductively isolated taxa) is often thought of as one of the most important forces driving diversification in plants and animals (Stuart & Losos, 2013). Although several animal studies (but few plant studies) suggest ECD, few of them present strong

evidence for its efficacy. In particular, Schluter and McPhail (1993) suggest six criteria that need to be fulfilled in order to show ECD and few studies have been able to fulfill more than three of these. In this chapter I investigate ECD in *Pelargoniums* visited by *P. longipennis*. I hypothesize that floral variation through ECD may be driven without any geographic variation in pollinator communities. This can occur because *Pelargoniums* all place pollen on the ventral surface of the flies' bodies. To reduce the negative effects of interspecific pollen transfer (IPT), plants may evolve differences in their reproductive parts when they occur in sympatry. This is expected to reduce the overlap of pollen placement on the bodies of pollinators. More specifically, I hypothesize if IPT is driving character displacement then 1) similarity in style length is likely to result in pollen overlap on the body of the pollinator and increase levels of IPT for sympatric species. 2) IPT will have negative consequences for one or more interacting species (i.e. IPT will cause reductions in seed set). If both of these predictions are met, there will be grounds to expect a pattern consistent with character displacement. More specifically, we predict that 3) style length of sympatric species will have diverged, whereas style length in allopatric populations may be frequently overlapping. 4) This pattern should occur repeatedly for several populations and species and should not be attributable to chance or ecological sorting.

Chapter 2

MATCHING FLORAL AND POLLINATOR TRAITS THROUGH GUILD CONVERGENCE AND POLLINATION ECOTYPE FORMATION

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ABSTRACT

Pollinator landscapes, as determined by pollinator morphology/behavior can vary inter- or intra-specifically imposing divergent selective pressures and leading to geographically divergent floral ecotypes. Assemblages of plants pollinated by the same pollinator (pollinator guilds) should exhibit convergence of floral traits because they are exposed to similar selective pressures. Both convergence and the formation of pollination ecotypes should lead to matching of traits among plants and their pollinators. We examine 17 floral guild members pollinated in all or part of their range by *Prosoeca longipennis*, a long-proboscid fly with geographic variation in tongue length. Attractive floral traits such as colour, and nectar properties were recorded in populations across the range of each species. The length of floral reproductive parts, a mechanical fit trait, was recorded in each population to assess possible correlation with the mouthparts of the local pollinator. Multiple regressions were used to determine whether pollinators or abiotic factors provided the best explanation for variation in floral traits, and pollinator shifts were recorded in extra-limital guild member populations. Nine of the 17 species were visited by alternative pollinator species in other parts of their ranges and these displayed differences in mechanical fit and attractive traits, suggesting putative pollination ecotypes. Pollinator shifts have resulted in geographically divergent pollinator ecotypes across the ranges of several guild members. However, within sites, unrelated plants pollinated by *P. longipennis* are similar in the length of their floral parts, most likely as a result of convergent evolution in response to pollinator morphology. Both of these lines of evidence suggest that pollinators play an important role in selecting for certain floral traits.

INTRODUCTION

The interaction between floral phenotype and morphology or behavioral preferences of pollinators can have a profound effect on plant fitness (Nilsson, 1988; Schemske & Bradshaw, 1999). Variation in certain aspects of floral phenotype, including colour or fragrance, may affect the attractiveness of a flower to a pollinator (Newman *et al.*, 2012) and these are often termed “attractive traits” (Ashman & Morgan, 2004). Other morphological traits such as perianth tube/spur length are unlikely to affect the attractiveness of flowers to pollinators, but instead influence the efficiency of pollen deposition or receipt through the fit between floral and pollinator morphology (Nilsson, 1988; Johnson & Steiner, 1997; Alexandersson & Johnson, 2002; Anderson *et al.*, 2010a; Boberg *et al.*, 2014). Such traits are frequently called “mechanical fit” traits (Muchhala, 2007). The influence on floral traits by the morphology and behavior of their pollinators frequently results in close matching between plant and pollinator traits (Anderson *et al.*, 2010b). The adaptive matching of plant and pollinator traits can generate divergent as well as convergent patterns of trait evolution.

Divergent patterns of trait evolution occur when geographic differences in pollinator morphology or behavior influence the direction and intensity of selection on plant traits. This drives the evolution of divergent floral morphologies, often identified as pollination ecotypes (Grant & Grant, 1965; Stebbins, 1970). Classically, ecotypic divergence occurs when a plant species is pollinated by morphologically or behaviorally different pollinators in different parts of its range (Grant & Grant, 1965; Robertson & Wyatt, 1990; Arroyo & Dafni, 1995; Johnson, 1997; Johnson & Steiner,

1997; Anderson *et al.*, 2010a; Forest *et al.*, 2013; Boberg *et al.*, 2014; Peter & Johnson, 2014; Sun *et al.*, 2014; Van der Niet *et al.*, 2014b). A natural extension to this “pollinator-shift” or “Grant-Stebbins” model of divergence (as described by Johnson, 2010), is the recognition of pollinator ecotypes which do not require pollinator shifts (Johnson, 2010; Ellis & Anderson, 2012). Examples of these often involve floral adaptations to intra-specific variation in the body parts of pollinators that are used to acquire rewards (Steiner & Whitehead, 1990b; Johnson & Steiner, 1997; Anderson & Johnson, 2008; Anderson & Johnson, 2009; Pauw *et al.*, 2009) or access oviposition sites (Thompson *et al.*, 2013). These studies suggest that ecotypic divergence could be founded on reciprocal adaptations (i.e. coevolution) between plants and pollinators (e.g. Anderson and Johnson, 2008; 2009; Pauw *et al.*, 2009; Thompson *et al.*, 2013), as well as through unilateral evolution, in which only the plant adapts to the pollinator and not *vice versa* (Anderson and Johnson, 2009; Boberg *et al.*, 2014).

Floral traits such as colour, scent or shape may converge among assemblages of plants due to selection imposed by a common pollinator. These groups of frequently unrelated, but similar looking flowers with a common pollinator are called ‘pollinator guilds’ (Fenster *et al.*, 2004). While it is tempting to think of the traits associated with guilds as being geographically homogenous, the morphology of some pollinators is known to be geographically variable and there are consequently examples of plant guilds in which traits that vary greatly between sites nevertheless converge within sites in response to selection from a geographically variable pollinator (Anderson and Johnson, 2009; Pauw *et al.*, 2009).

One problem with trait matching studies is that it is often difficult to distinguish the causes of the observed patterns (Nuismer *et al.*, 2010). For example, trait matching could result from adaptation of a plant to a pollinator, adaptation of a pollinator to a plant, or a combination of the two. However, multiple studies show that the precision of the fit between pollinator mouthparts and floral tubes has a strong effect on plant fitness, suggesting that tube length is often an adaptive trait (Nilsson, 1988; Muchhala & Thomson, 2009; Pauw *et al.*, 2009). This does not negate the possibility that pollinator mouthparts may be simultaneously adapting to floral mouthparts (e.g. Pauw *et al.*, 2009). Alternatively, the matching of pollinator and floral parts may also be a result of abiotic factors that affect both interacting species similarly. Hence altitude and latitude are sometimes used as surrogates of the abiotic environment to determine whether they have an effect on the trait variation of interacting species (Anderson & Johnson, 2008).

Here, we study a recently discovered guild of plants that are pollinated throughout, or in parts of their range, by the long-proboscid Nemestrinid fly *Prosoeca longipennis* Loew. Our observations suggest that, like several other long-proboscid flies, *P. longipennis* displays geographic variation in proboscis length. The floral members belonging to the *P. longipennis* pollination guild have been characterized as having deep corolla tubes, no odor discernible to humans, and a cream, salmon, or pink perianth contrastingly marked with red streaks or spots (Manning & Goldblatt, 1995; Goldblatt & Manning, 2000b). The *P. longipennis* guild is separated from most other morphologically similar long-proboscid fly pollination guilds by flowering in the Austral autumn months rather than in the spring or summer, when most other long-proboscid fly pollinated plants are in flower.

Manning and Goldblatt (1995) identified five species of plants as actually or putatively visited by *P. longipennis* at two sites. Here we report on several additional *P. longipennis* sites and add substantially to the guild of plants pollinated by this species. We measured floral and pollinator traits at each site to determine whether they are geographically variable and used multiple regression to identify the factors associated with variation in floral traits. We predict that 1) pollinator mouthpart lengths and associated floral traits (e.g. tube length) will be closely matched as a result of adaptation to the local pollinator morphology. 2) This should manifest itself as strongly correlated patterns of geographic covariation between pollinator and plant morphology, and that the best explanatory variable for variation in plant morphology should be the morphology of the pollinator.

MATERIALS AND METHODS

Study species and sites

Prosoeca longipennis (Nemestrinidae) is a large species of long-proboscid fly (Fig. 2.1) that forages most actively from morning to early afternoon between 08:00 AM and 13:00 PM, depending on the weather. To investigate trait covariation between *P. longipennis* flies and flowers, *P. longipennis* flies and floral guild members were studied across 14 different study sites in the Western and Eastern Cape provinces of South Africa (see Fig. 2.2). The range of the fly extends across fynbos and grassland biomes, with the bulk of the known range in the fynbos biome. The fynbos biome is characterized climatologically by winter or aseasonal rainfall and ecologically by very high floral diversity within a relatively small number of locally diverse families, including Proteaceae, Orchidaceae, Iridaceae and Ericaceae

(Goldblatt & Manning, 2000a). A small part of the eastern range of *P. longipennis* falls within temperate grassland, characterized by summer rainfall and a high diversity of geophytes. The vegetation of both of these biomes is fire-adapted.

Prior to this and two other recent studies (de Merxem *et al.*, 2009; Ros, 2010), *P. longipennis* had been positively identified from only a few populations. Over ten years (2004–2013) we increased the number of known populations to 14 by visiting localities with autumn (February–March) flowers that fitted the preliminary guild description of Manning and Goldblatt (1995).

POLLINATION GUILD: SPECIES COMPOSITION AND DISTRIBUTION

We aimed to identify as many plant taxa as possible that are consistently visited by *P. longipennis* in at least part of their range, thus enabling us to describe the guild more fully. Plants pollinated by more than one functionally different pollinator within a site were not included as guild members. At each site we identified local flower species visited by the fly through direct observation. Pollination by *P. longipennis* was confirmed by observing the fly visiting a particular flower and making contact with the reproductive parts of that flower.

Measurements of morphological traits

At each site, specimens of *P. longipennis* were caught by net and killed by either lethal injection of absolute ethanol using a diabetic syringe or by gassing with potassium cyanide fumes. The average functional proboscis length, i.e. the maximum length that an individual fly can extend its proboscis, of between one and seven flies

(see supplementary Table S2.1) per population was measured using digital calipers (methods outlined in Anderson *et al.*, 2005).

For each plant species, we attempted to quantify the traits that were putatively adapted to fly proboscis length or to which fly proboscis length could be adapted. Most long-proboscid fly guild members have long, slender perianth tubes or spurs that accommodate the flies' proboscis from tip to head (Goldblatt and Manning, 2000). Pollen placement on the fly is determined by the position of the anthers in relation to the mouth of the nectar tube or spur. In most species the anthers are situated near to the mouth of the tube. To quantify the portion of the flower that is putatively adapted to proboscis length, we measured only the part of the flower that accommodates the proboscis. This measurement was from the site of nectar secretion (usually the top of the ovary in epipetalous species) to the top of the nectar tube. The top of the nectar tube was judged to be the position where the width of the nectar tube widened rapidly/or opened completely, a point that corresponds to the base of the tepals or petals in gamopetalous species and to the point at which the petals spread in polypetalous species. From seven to 70 individuals per species were measured in this manner (see supplementary Table S2.2 for replication details of each species). *Nerine humilis* (Jaqu.) Herbert (Amaryllidaceae) is unusual in this guild as it lacks a perianth tube. Instead the highly elongated filaments and style are located such that the stamens and stigmas make contact with the fly precisely on its abdomen. The measurement that corresponds to fly proboscis length in *Nerine humilis* is the total length of the stigma–nectary distance, minus the total body length of the fly (see Anderson and Johnson (2009) for details and explanations of this). Measurements of *Nerine humilis* were taken only when flowers were in female phase, after the stigma

has curved into the mature position, occupying the place where the anthers were previously presented.

Nectar volume and concentration was measured in 10–25 flowers per species at each locality (one flower per randomly chosen inflorescence) using a 1–5 μ l graduated micropipette (Drummond Scientific Company, Broomall, Pennsylvania, USA) and a 0–50% Bellingham-Stanley refractometer (Bellingham and Stanley, Tunbridge Wells, UK) measured in the afternoon. Nectar volumes that were too small to be measured individually were combined and averaged over several individuals. Replication details can be found in Table 1. Total range and flowering time for each plant species are from Goldblatt and Manning (2000b).

The principal attractive surface (the dominant colour of the flower) of floral guild members was objectively assessed using a spectrometer (Dunedin – Florida), measured across the range of 300–700nm, and averaged from five samples from different individuals. Although multiple guild members were assessed in this manner, we present only a single example from each plant family. Samples were chosen to represent species that appeared to be most different in colour to the human eye, thus capturing a wide range of the colour variation utilized by *P. longipennis*. In addition to using spectral reflectance to visualize colour differences, we evaluated floral colour for each species in each population as visualized by the eyes of the primary author (EN). These differences included the dominant colour of the flower as well as the colour of nectar guides when present.

POLLINATION ECOTYPES (SHIFTS)

We investigated the existence of ecotypes in instances where published literature suggested other pollinators in extra-limital populations or in instances where floral characteristics of guild members pollinated by *P. longipennis* were unusual for the species. Putative pollination ecotypes were identified in plant species that extended beyond the known geographical range of *P. longipennis* and that exhibited intraspecific variation in attractive (e.g. colour) or mechanical fit traits (e.g. tube or stigma length) that correspond with putative shifts in pollinator species. We did not make additional field observations in instances where previously published research identified extra-limital populations pollinated by different pollinators. In such instances we compared colour and tube length among populations from published data, and where that was not available, herbarium records were used. In instances where nothing was known about pollinators outside of the *P. longipennis* range, pollinator observations were made in single putative ecotype populations. Pollinator visitations were recorded for a single day of observation at temperatures averaging above 20 degrees Celsius. Only visitors that made contact with the reproductive parts of the plant were recorded as pollinators (Table S2.3 & S2.4). In these cases, data on floral morphology were collected directly in the field. Differences in tube length among populations pollinated by different pollinators were assessed using an independent samples t-test. In instances where more than one *P. longipennis* pollinated population was known, measurements were pooled from all known *P. longipennis* populations as a comparison to the putative extra-limital ecotypic population. The sources and replication details of the ecotype data are provided in supplementary Table S2.2 and S2.3.

Analysis of co-varying traits and population level trait convergence

To determine possible covariance between pollinator and plant morphology, we performed an ordinary least squares (OLS) regression analysis based on mean fly proboscis length at each site and the grand mean (mean of means) of all *P.*

longipennis pollinated flowers within a site. In addition, we ran a multiple regression on the grand mean of rewarding flowers at each site using latitude, longitude, altitude and fly tongue length as predictive variables in order to determine if surrogates for the abiotic environment might also influence floral variation. To investigate whether floral tube length was a predictor of fly tongue length, we performed a similar analysis using the grand means of rewarding flowers at each site as well as latitude, longitude and altitude as predictor variables.

Multiple regressions assume that data points used within the regression are independent of each other. However, if morphology is structured by gene flow or common descent, then populations with similar tube lengths will not be independent of one another. Since gene flow and relatedness are often closely associated with geographic proximity (isolation by distance); the independence of data points can be tested by correlating pairwise geographic differences between populations with pairwise morphological differences between populations (*see* Anderson and Johnson, 2008). If a non-significant relationship exists, then trait values in each population are probably influenced by local adaptation. An alternative option would be to account for phylogenetic relatedness in the regression analysis. However, for many of the plant groups in the *P. longipennis* pollination guild, we lack this information (Hansen *et al.*, 2000). To test for the independence of sites, we used a Mantel test (1000 permutations) in NTSYS (Rohlf, 1992) of pairwise proboscis and tube length

differences versus pairwise differences in geographic proximity. All statistical analysis, other than Mantel tests were computed in SPSS v. 20.0 (IBM Inc.).

RESULTS

Seventeen plant species from four different families were pollinated by *P. longipennis* at one or more sites (Table 2.1). Some of these plants were also occasionally visited by other functionally identical species of *Prosoeca* with very similar proboscis lengths to *P. longipennis*. To the human eye, flowers pollinated by *P. longipennis* are typically white, cream or pink in colour, with a single pale blue species (*Wahlenbergia guthrie*) (Table 2.2, Fig 2.3). Nectar guides among guild members were predominantly various shades of purple to red (Table 2.2). *Gladiolus martleyi* was exceptional in having yellow and red nectar guides (Table 2.2). Despite apparent visual differences to the human eye (Table 2.2, Fig 2.3), all species including *W. guthrie* have a strong reflectance peak in the range 350 – 450nm (Fig 2.4). Nectar volume was highly variable within the guild (between 0.98 μ l and 7.93 μ l), as was sugar concentration (between 13.39% and 35.24%) (Table 2.1). None of the species was evidently fragrant to the human nose. Flowering of guild members throughout their ranges has been recorded from November to June, although *P. longipennis* has only been recorded on the wing from late February to May. In addition, the range of the fly tends to be very patchy and does not correspond closely with the ranges of many guild members.

POLLINATION ECOTYPES

Nine of the 17 species pollinated by *P. longipennis* are visited by species other than *P. longipennis* in different parts of their geographic range. We recorded birds and

several insect orders, including Diptera, Hymenoptera and Lepidoptera as additional visitors to guild members. Eight of the nine plant species display morphological differences in tube length that correspond to different pollinators (Table 2.3), while four of those nine species also show colour variation associated with different pollinators (Table 2.3). In summary, around half of the seventeen species that we identified as belonging to the *P. longipennis* guild show evidence of the existence of pollination ecotypes.

COVARIATION AND CONVERGENCE

The floral tube length of all guild members at each site (grand mean) correlated strongly with the average proboscis length of the corresponding local flies ($R^2 = 0.71$, $F = 33.37$, $P < 0.0001$, Fig. 2.5 & 2.6). The multiple regression model explaining proboscis length variation was significant ($R^2 = 0.68$, $F = 6.75$, $P < 0.015$, Table 2.4), with tube length as the only significant predictive variable ($\beta = 3.2$, $P = 0.014$, Table 2.4). The multiple regression model explaining tube length was also significant ($R^2 = 0.79$, $F = 11.01$, $P < 0.004$, Table 2.4) and similarly the only significant predictive variable was proboscis length ($\beta = 0.63$, $P = 0.014$, Table 2.4), with latitude, longitude and altitude as non-significant explanatory variables. Mantel tests revealed that pairwise differences in mean proboscis length (mantel $r = -0.29$; $P > 0.72$) were not significantly structured by geographical distance.

DISCUSSION

***PROSOECA LONGIPENNIS* GUILD CHARACTERISTICS**

Our results support the existence of a guild of autumn flowering plants pollinated almost exclusively by the Nemestrinid fly *P. longipennis* in parts of their range (occasionally in conjunction with other functionally equivalent long-proboscid flies). We have identified 17 plant species from four different families (Amaryllidaceae, Iridaceae, Campanulaceae, and Geraniaceae) as members of this guild (Table 2.1). Although *Protea punctata* Meisn. (Proteaceae) also has traits that fit the *P. longipennis* syndrome, we hesitate to include it in this guild because it is also visited frequently by the functionally different butterfly pollinator *Aeropetes tulbaghia* in the same populations where *P. longipennis* appears to be an important pollinator (Johnson *et al.*, 2012).

Flowers belonging to the guild have similar traits to species in other South African long-proboscid fly pollination guilds, notably pale coloration with dark nectar guides, long nectar tubes, and lack of evident floral fragrance. *P. longipennis* pollination guild members have a strong spectral peak between 350–450 nm, appearing pink, peach or white to the human eye (Table 2.2; Fig 2.4.). These flowers are similar in colour to guild members of both the *Moegistorhynchus-Philoliche* guild from western South Africa (Goldblatt and Manning, 2000a) and the *Prosoeca ganglebauri* pollination guild in the eastern half of South Africa (Goldblatt and Manning, 2000a). Pale blue flowered *Wahlenbergia guthrie* is unusual among the pink and white guild members (see Fig 2.3). Significantly, however, the reflectance spectrum and spectral peak (~430 nm) of this species is very similar to other guild members, suggesting that peaks at this wavelength may be important in attracting *P.*

longipennis (Fig. 2.4). Other long-proboscid fly guilds with predominantly pink colouring also include occasional blue members, for example the bright blue *Nivenia stenosiphon* in the predominantly white–pink *P. ganglbaueri* guild (Goldblatt and Manning 2000a) and blue *Agapanthus campanulatus* in the predominantly pink *Philoliche aethiopica* guild (Jersakova *et al.*, 2012). Although the presence of blue flowers in these guilds may appear out of place to the human visual system, long-proboscid Tabanid fly's *Philoliche aethiopica* are unable to distinguish between blue and pink when offered preferences in acetate model inflorescences (Jersakova *et al.*, 2012).

Like the flowers of many other long-proboscid fly pollination guilds, all but two species pollinated by *P. longipennis* have nectar guides. The presence of nectar guides is known to substantially increase visitation rates of long-proboscid flies at short distances (Hansen *et al.*, 2011) but has no effect on long distance attraction of flies. This link between fly foraging behavior and nectar guides on floral fitness is presumably similar in the *P. longipennis* guild.

Although the similarities in floral colour and markings suggest that flowers have converged on colour preferences of *P. longipennis* it is also possible that flower colour and the presence of nectar guides is simply a conserved ancestral trait in some taxa. For example, many members of the genus *Nerine* and *Pelargonium* have peach, pink or white flowers, suggesting that this trait is frequently not a response to selection specifically by *P. longipennis*. However putative ecotypes frequently display differences in floral colour (Table 2.3), suggesting that pollinators do select for floral colour. For example, bird pollinated populations of *T. antholyza* are red

whereas fly pollinated *T. antholyza* populations are cerise pink. While these colour shifts suggest pollinator selection on floral colour, it is impossible to determine the direction of those shifts without an accurate phylogeny (Valente *et al.*, 2012). Furthermore, selection experiments or reciprocal translocation experiments are needed to show that such traits are indeed locally adapted (e.g. Newman *et al.*, 2012).

The nectar concentrations and volumes of *P. longipennis* guild members fall within the range of other long-proboscid fly pollinated plants in South Africa (*see* Goldblatt and Manning, 2000) as well as plants pollinated by large bodied Hymenoptera (Goldblatt *et al.*, 1998; Goldblatt *et al.*, 2001). The variability of these traits suggests that nectar characteristics are not strongly selected on by *P. longipennis*. The lack of discernible floral scent in *P. longipennis* floral guild members is characteristic of other South African long-proboscid fly pollination guilds (Goldblatt and Manning, 2000a).

The diagnostic feature of the *P. longipennis* guild is that many of the plants reach their flowering peaks in the autumn months, when relatively few other flowering plants are in bloom. For some guild members at least, flowering time appears to be conserved. For example, most members of Western Cape Amaryllidaceae (including *Nerine* and *Cyrtanthus*) as well as the iridaceous genus *Tritoniopsis* flower in the autumn months. In such cases, autumn flowering is unlikely to be a specific adaptation to *P. longipennis* pollination but is likely to have been an important trait, or preadaptation, that facilitated entry into the guild.

Based on distribution, autumn flowering, lack of scent, long tube lengths, and perianth colour, we predict that several other *Watsonia* species are likely to be visited regularly by *P. longipennis*, notably *W. galpinii*. While *Brunsvigia striata* is visited by carpenter bees in parts of its range (see photograph in Vlok and Schutte-Vlok, 2010), this species may also be visited by *P. longipennis* in parts of its range, potentially forming ecotypes. We also predict that the autumn-flowering *Gladiolus dolichosiphon*, endemic to the Little Karoo, is pollinated primarily or exclusively by *P. longipennis*.

ECOTYPES THROUGH POLLINATOR SHIFTS

The high number of morphologically divergent pollination ecotypes in this study system provides evidence suggesting that variation in floral morphology frequently reflects adaptation to local pollinators. Eight of the nine plants with alternative pollinators outside of the *P. longipennis* range exhibited ecotypic variation associated with tube length, perianth colour, or both. For example (Table 2.3), plants of *Pelargonium dipetalum* from localities where *P. longipennis* occurs have long nectar tubes (mean \pm SE = 69.47 \pm 2.29 mm) with cream to white flowers, whereas extra-limital populations have pink flower with short nectar tubes (mean \pm SE = 11.58 \pm 0.52 mm) that attract an array of smaller, short proboscis insect pollinators (Manning and Goldblatt, 1995).

The importance of pollinator shifts in generating morphological and genetic variation has also been suggested in other long-proboscid fly guilds. Thus, in the *Moegistorhynchus longirostris* pollination guild, pollinator shifts have been identified in two different plant groups (Johnson and Steiner, 1997; Pauw *et al.*, 2009). Within

the *Disa draconis* (Orchidaceae) complex and in *Lapierousia anceps* (Iridaceae), shifts between shorter proboscis Tabanid flies and Nemistrinid flies with relatively longer proboscides have generated putative ecotypes that match the distributions of their pollinators. Selection on floral tube length was also demonstrated through artificial manipulations on orchid spur length (Johnson and Steiner, 1997) as well as by calculating selection gradients in response to pollinators with variable proboscis lengths (Pauw *et al.*, 2009). Selection of floral tube length on fly proboscis length also suggests that these are coevolving (Pauw *et al.*, 2009.)

The existence of a near-complete phylogeny (*see* Valente *et al.*, 2012) for the genus *Gladiolus* enables us to make some conclusions about how pollination by *P. longipennis* originated in guild members of this genus. *Gladiolus* is exceptionally well represented in long-proboscis fly systems (Goldblatt and Manning, 2000a) and all four species identified in our study are included in the Valente *et al.* (2012) phylogeny. These four species, *G. martleyi*, *G. oppositiflorus*, *G. engysiphon*, and *G. bilineatus*, are located in very different parts of the phylogeny and entry into the *P. longipennis* pollination guild seems to have taken place independently at least three times, with only *G. martleyi* and *G. engysiphon* members of a common clade. Unfortunately, the relationships within the major clades (which support multiple pollination modes) are typically unresolved and so in most cases it is difficult to determine the ancestral states of species pollinated by *P. longipennis*. Bee pollination is evidently ancestral in most major *Gladiolus* clades, and Valente *et al.* (2012) identified *G. martleyi* as being bee pollinated. The longer tubed individuals of this species that were studied by us were exclusively pollinated by long-proboscis flies. Significantly all *G. martleyi* flowers (including the long-proboscis fly pollinated

population) have yellow nectar guides, which is very common among bee pollinated *Gladiolus* species, but not long-proboscid fly pollinated *Gladiolus* species (Goldblatt *et al.*, 1998). Furthermore, the colour of nectar guides in all other *P. longipennis* pollinated flowers were reddish. Although longer than in other *G. martleyi* populations, the floral tubes in the study population were still much shorter than the proboscides of the visiting flies and appear as an outlier in Figure 2.5. These lines of evidence suggest a recent shift from bee pollination to pollination by *P. longipennis* in this population of *G. martleyi*, with incomplete adaptation in tube length and perianth markings. The other extreme outlier in this study, *G. bilineatus*, has a tube which is substantially longer than the tongue of its pollinator. Mismatches in this direction in pollination mutualisms probably reflect an asymmetry in the selection pressures acting on floral versus pollinator traits (Anderson *et al.*, 2010b).

Fine-scale geographic trait covariation in the P. longipennis guild

The similarity in tube length among guild members within a site, as well as the strong correlation between mean proboscis length of the fly pollinators and the grand mean of the guild members per site, suggest that adaptation is taking place at fine geographic scales (Table 2.4; Fig 2.5 and 2.6). Similar patterns of trait covariation have also been observed in other pollination mutualisms where plants are probed by pollinators with long legs or proboscides (Steiner & Whitehead, 1990b; Anderson & Johnson, 2008; Anderson & Johnson, 2009; Cosacov *et al.*, 2014). These observations suggest that close trait matching between pollinators and plants may be a common and general trend (as suggested Anderson *et al.*, 2010b). Close matching between traits is expected in mutualisms because traits in such relationships tend to converge in order to maximize the mutual gains of the interaction (Thompson, 2013). In

theoretical contrast, strong trait matching is not expected as the norm in antagonistic relationships because one organism by definition should be evolving mechanisms to avoid the interaction through the mismatching of traits (Thompson, 2013). While there are not yet enough empirical studies to support the idea of general differences in the strengths of trait matching between mutualism and antagonism, the only study that attempts to make a comparison finds little difference between the two kinds of interaction (Anderson *et al.*, 2010b).

Several selection studies show that in other similar systems, elongated floral traits are an adaptive response to the elongated traits of their pollinators (Nilsson, 1988; Johnson and Steiner, 1997; Anderson and Johnson, 2008; 2009; Pauw *et al.*, 2009). We therefore consider it likely that tube length variation among the *P. longipennis* guild members represents similar adaptations to the geographic variation of pollinator proboscis length. What is not clear is whether tube length variation is the result of reciprocal adaptation (i.e. coevolution), as has been demonstrated in a very similar long-proboscid fly system by Pauw *et al.* (2009). This study, and the fact that long tubed flowers form the only food source for these flies, suggests that long tubed flowers may also select on proboscis length and that in many instances, tube lengths of entire guilds and fly tongue lengths have probably coevolved (as suggested by Anderson and Johnson, 2009; Pauw *et al.*, 2009).

An alternative to coevolution is that exaggerated floral traits in the *P. longipennis* pollination guild evolve through one-sided or unilateral evolution in which the flowers adapt to the flies but not *vice versa*. For example, a plant species such as *N. humilis* is unlikely to select strongly on fly proboscis length because it

lacks a nectar tube and is therefore unable to exert selective pressure on the mouthparts of the flies to match or exceed the reproductive parts of this species since the nectar is equally accessible to insects with very short mouthparts. In an example of Batesian mimicry, Anderson *et al* (2005) and Anderson and Johnson (2009) also showed unilateral selection operating on the spur length of a nectarless mimetic orchid pollinated by a closely related long-proboscid fly *P. ganglebauri*.

Conclusion

We conclude that pollinators play an important role in driving both the divergence and convergence of floral traits through plant adaptations to pollinator morphology. The role of pollinators in driving floral evolution may be especially important in systems such as this one, in which plants are specialized for one or two functionally identical pollinators at a site. We predict that close morphological matches between specialized plants and their pollinators is the norm and that significant trait correlations will be evident when pollinators are geographically variable. Strong correlations between pollinator and plant traits bear testimony to the power of floral morphology in predicting the morphological features of pollinators and *vice versa*, lending support to the concept of pollination syndromes (the idea that plants pollinated by a common pollinator should share trait similarities).

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Table. 2.1. Average floral nectar measurements \pm SE, species range and flowering phenology of *P. longipennis* pollinated populations of plants. Numbers in parentheses indicate the number of individuals sampled. Locality range data has been modified from Goldblatt and Manning (2000b). Where abbreviations LB: Langeberg, KM: Karoo Mountain; AP: Agulhas Plain, SW: South West, NW: North West and EC: Eastern Cape has been used to indicate the phylogeographic region of occurrence. See Figure 1 for graphical explanations of regional positions.

Species	Family	Nectar concentration (%)	Nectar volume (ml)	Range	Flowering months
<i>Cyrtanthus leptosiphon</i>	Amaryllidaceae	18.63 \pm 1.53 (10)	5.60 \pm 0.99(10)	LB	Feb - Mar
<i>Geissorhiza fourcadei</i>	Iridaceae	-	-	KM	Mar
<i>Gladiolus bilineatus</i>	Iridaceae	27.17 \pm 1.33(9)	2.98 \pm 0.72(9)	LB	Mar- Apr
<i>Gladiolus engysiphon</i>	Iridaceae	23.12 \pm 0.36(14)	1.92 \pm 0.31(14)	LB	Mar - Apr
<i>Gladiolus martleyi</i>	Iridaceae	26.65 \pm 2.03(3)	-	LB, SW, NW, AP	Febr - May

<i>Gladiolus oppositiflorus</i>	Iridaceae	29.47 ±1.19 (20)	7.83 ± 1.04(20)	EC	Feb - Mar
<i>Nerine humilis</i>	Amaryllidaceae	35.24±2.86 (17)	1.47±0.08 (17)	LB, NW, SW, AP, KM, SE	Mar - Jun
<i>Pelargonium carneum</i>	Geraniaceae	22.64±3.48 (11)	1.91±0.44 (11)	LB, SW, EC	Mar
<i>Pelargonium dipetalum</i>	Geraniaceae	20.92 ±2.07(17)	2.73±0.87 (17)	LB, SW,	Feb - Mar
<i>Pelargonium laevigatum</i>	Geraniaceae	-	-	KM, SE, EC	Mar
<i>Pelargonium pinnatum</i>	Geraniaceae	13.39±1.17 (14)	1.81±0.38 (14)	LB, SW, KM	Mar - Jun
<i>Pelargonium reniforme</i>	Geraniaceae	20.48± 0.92(20)	2.62 ±0.39 (20)	EC	Mar
<i>Pelargonium tetragonum</i>	Geraniaceae	-	-		
<i>Pelargonium zonale</i>	Geraniaceae	-	-		
<i>Tritoniopsis antholyza</i>	Iridaceae	20.78 ± 1.1(20)	0.98 ± 0.24(20)	NW, SW, AP, KM, LB, SE	Nov - Apr
<i>Tritoniopsis revoluta</i>	Iridaceae	29.38± 1.01(20)	6.19± 0.85(20)	AP, KM, LB	Mar - May

<i>Wahlenbergia guthrie</i>	Campanulaceae	-	-	KM	Jan - March
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Table. 2.2. Flower colour of floral guild members present at each site as seen by human vision. Crosses in the center of cells refer to the presence of nectar guides and their colour as perceived by human vision.

Species	Localities													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>Cyrtanthus leptosiphon</i>									X					
<i>Geissorhiza fourcadei</i>														
<i>Gladiolus bilineatus</i>						X								
<i>G. engysiphon</i>						X			X					
<i>G. martleyi</i>									X					
<i>G. oppositiflorus</i>							X							
<i>Nerine humilis</i>	X								X					
<i>Pelargonium carneum</i>			X			X			X					
<i>P. dipetalum</i>									X					
<i>P. laevigatum</i>	X		X											
<i>P. pinnatum</i>						X			X					
<i>P. reniforme</i>								X						
<i>Pelargonium tetragonum</i>	X													
<i>Pelargonium zonale</i>	X													
<i>Tritoniopsis antholyza</i>		X		X	X									
<i>T. revoluta</i>										X	X	X	X	X
<i>Wahlenbergia guthriei</i>														

Table. 2.3. Pollination ecotypes of *P. longipennis* pollination guild members, significant differences in floral traits associated with different pollinators have been highlighted in bold, as have colour difference as perceived by the primary author (EN). Reproductive traits for *Nerine humilis* lacks a long floral tube, consisting only of extended stamens and stigmas. Significant differences in tube length traits are indicated by asterisks: $P^* < 0.05$; $P^{**} < 0.001$; $P^{***} < 0.0001$. Refer to abbreviations in (Table 1) on the ranges of the different ecotypes and the summary of pooled tube length data for *P. longipennis* guild members from Table S2.

<i>Prosoeca longipennis</i> ecotype					Ecotype other than <i>P. longipennis</i>				
species	colour	nectar guide color	mean tube length / trait (mm)	range	Pollinator group	colour	nectar guide colour	mean tube length/ trait (mm)	ecotype range
<i>Gladiolus</i>	pink	yellow and	22.47±0.50(7)	LB, SW, NW,	Various	pink	yellow and red	***11.22±0.61(12)	SW, NW, AP
<i>martleyi</i>		red		AP	Hymenoptera				
<i>Nerine humilis</i>	pink	red	43.14±0.53(87)	LB, NW, SW, AP, KM, SE	Various Hymenoptera & Diptera,	pink	absent	***27.44±0.74(34)	NW, SW, AP, KM, SE
<i>Pelargonium</i> <i>carneum</i>	cream	salmon - red	50.43± 2.11(54)	LB, SW, EC	Butterflies: <i>Princeps</i> <i>demodocus</i>	cream	salmon - red	42.06±1.30(33)	EC, SE

<i>Pelargonium dipetalum</i>	white	salmon	69.47±2.29(19)	LB, SW,	Various Hymenoptera and Diptera	pink	salmon	***11.58±0.52(25)	SW
<i>Pelargonium laevigatum</i>	white	red	24.87±0.58(28)	KM, SE, EC	Other Nemestrinid flies	pink	red	***12.35±0.56(10)	EC
<i>Pelargonium reniforme</i>	purple	red	41.80±0.89(70)	EC	Other Nemestrinid flies	purple	red	***24.15±0.83(72)	EC
<i>Tritoniopsis antholyza</i>	pink	white	36.9±0.58 (56)	NW, SW, AP, KM, LB, SE	Sunbirds	red	salmon	***27.90±0.80(11)	NW, SW, AP, KM, LB, SE
<i>Tritoniopsis revoluta</i>	pink	white and red	65.46±0.71(115)	AP, KM, LB	Various Hymenoptera and Diptera	pink	white and red	***22.50±0.71(128)	KM, AP
<i>Wahlenbergia guthrie</i>	blue	absent	27.85±0.87(17)		Various Hymenoptera	blue	absent	***10.78±0.54(16)	KM

Table. 2.4. Multiple regression model of the grand means of corolla tube/style lengths and mean fly proboscis lengths from 14 localities, that determine the relationship between these putatively adaptive traits together with surrogates for environmental predictor variables (latitude, longitude and altitude).

Response variable	Standardized partial regression coefficients (β)					model		
	proboscis length	corolla length	latitude	longitude	altitude	R^2	F	P
proboscis length		3.2*	-0.014	0.15	0.097	0.68	6.75	0.015
corolla length	0.63*		-0.13	-0.097	-0.47	0.79	11.01	0.004

$P^* < 0.001$

Table. S2.1. Mean proboscis lengths with standard errors, and sample sizes (n) of *P. longipennis* including latitude, longitude and altitude from each locality.

site	average proboscis length (mm)	latitude	longitude	altitude (m)
1	25±0.00(5)	33.41	22.51	1907
2	37.4±1.51(3)	33.44	23.44	1720
3	41±0.91(4)	33.76	23.15	988
4	43.25±2.13(6)	34.03	21.45	164
5	44.7±0.25(2)	30.12	28.71	2400
6	48.5±2.55(2)	32.96	25.22	465
7	48.5±2.10(4)	34.02	20.60	152
8	62±0.00(1)	33.94	21.77	560
9	63±2.00(2)	33.96	21.22	502
10	65.4±1.23(7)	33.93	21.20	260
11	68.5±0.75(6)	33.92	20.74	458
12	69.2±0.00(1)	33.98	20.71	350

Table S2.2 Average tube lengths with standard errors, including the numbers of individuals sampled (n) to generate these averages.

species	site											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>Cyrtanthus leptosiphon</i>								58.30 ± 2.61 (7)				
<i>Geissorhiza fourcadei</i>	21.38 ± 0.77 (8)											
<i>Gladiolus bilineatus</i>				71.00 ± 3.27 (10)								
<i>Gladiolus engysiphon</i>				64.67 ± 1.67 (15)				70.15 ± 1.03 (20)				
<i>Gladiolus martleyi</i>								22.47 ± 1.05 (7)				
<i>Gladiolus oppositiflorus</i>					35.34 ± 0.86 (20)							
<i>Nerine humilis</i>								42.65 ± 0.87 (26)				

<i>Pelargonium carneum</i>	34.61 ± 0.47 (21)	64.18 ± 2.10 (17)	56.24±1. 22(17)					
<i>Pelargonium dipetalum</i>			69.47 ± 2.29 (19)					
<i>Pelargonium laevigatum</i>	24.86 ± 0.58 (28)							
<i>Pelargonium pinnatum</i>		51.8 ± 1.76 (18)	63.21 ± 1.44 (19)					
<i>Pelargonium reniforme</i>			41.80 ± 0.89 (70)					
<i>Tritoniopsis antholyza</i>	30.5 ± 1.32 (4)	38.85 ± 1.14 (23)						
<i>Tritoniopsis revoluta</i>				56.64 ± 0.86 (20)	63.22 ± 1.13 (25)	68.00 ± 1.10 (30)	69.18 ± 1.24 (20)	69.6 ± 1.93 (20)
<i>Wahlenbergia guthrie</i>	27.85 ± 0.87 (17)							

Table S2.3: Sample sizes of pollination ecotype flower measurements from the field (F) as well as measurements from herbarium specimens (H). See references below for origin of pollination ecotype data.

Species	Sunbird	Generalist insects (Hymenoptera and Diptera)	Generalist Hymenoptera	Butterflies (Nymphalidae)	Other long- proboscid flies
<i>Gladiolus martleyi</i>			12(H) ²		
<i>Nerine humilis</i>		34(F) ⁵			
<i>Pelargonium carneum</i>				33(H) ³	
<i>Pelargonium dipetalum</i>		25(F) ⁵			
<i>Pelargonium laevigatum</i>		10(H) ³			10(H) ³
<i>Pelargonium pinnatum</i>		12(H) ⁵			
<i>Tritoniopsis antholyza</i>	7(F) ⁵				
<i>Tritoniopsis revoluta</i>					128(F) ⁴
<i>Wahlenbergia guthrie</i>		16(F) ⁵			

References:, Goldblatt *et al.*, 2001 (2), Struck, 2007 (3), Ross *et al.*, 2012 (4).ELN (Ethan Newman) personal observation/observations (5)

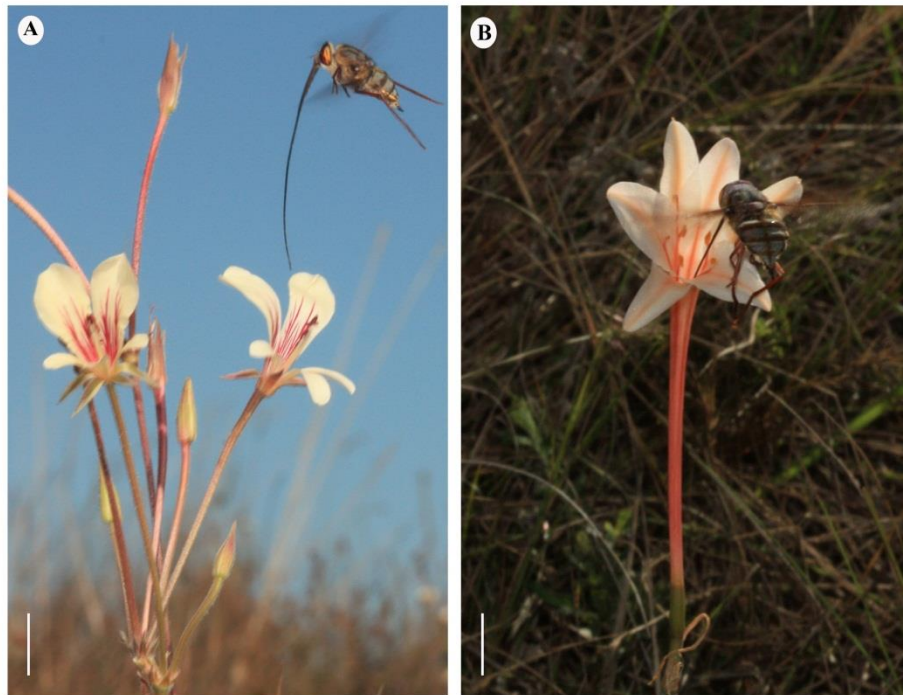


Figure. 2.1. (a) The long-proboscid fly *Prosoeca longipennis* visiting *Pelargonium carneum* (Geraniaceae) and (b) *Cyrtanthus leptosiphon* (Amaryllidaceae). Scale = 10mm

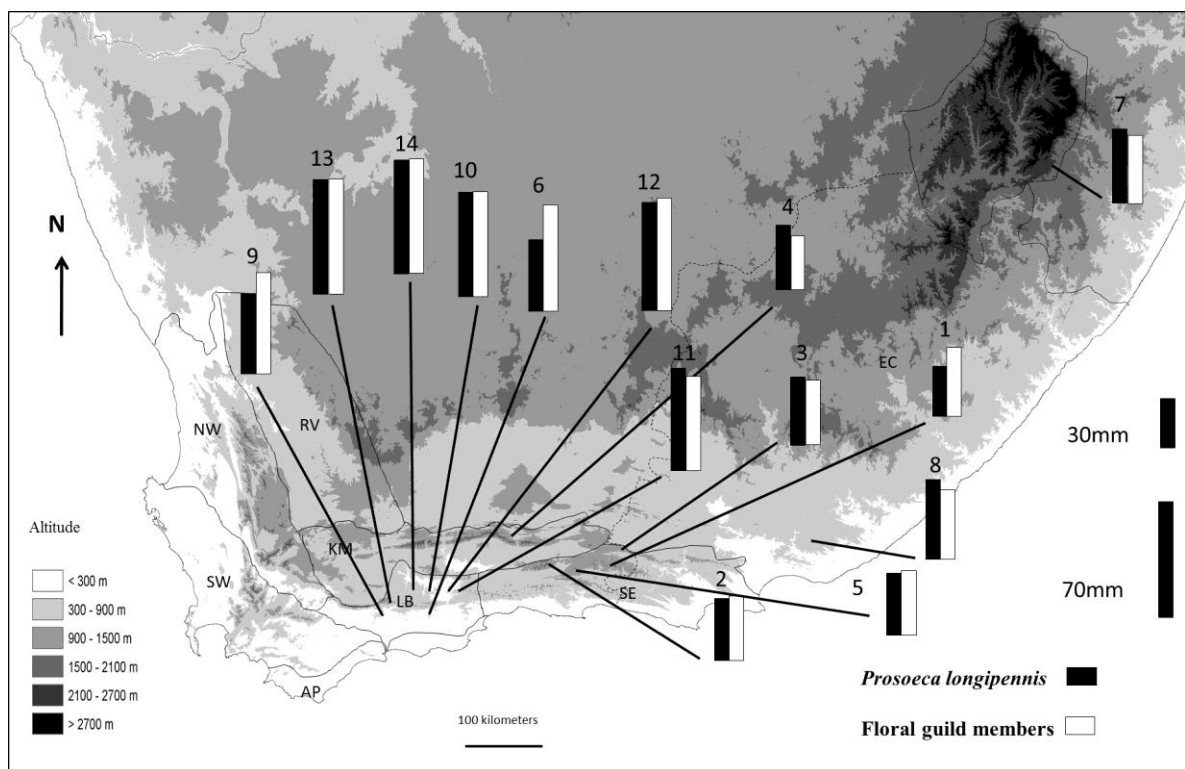


Figure. 2.2. Geographic variation in tube and proboscis length of *P. longipennis* and the flowers that it pollinates across 14 different localities. Black columns represent mean fly proboscis length and white columns represent grand means of all floral guild members within a site. Numbers refer to different populations and these correspond to Figure 5 as well as to the tables throughout the manuscript. The phytogeographical regions of the Cape Floristic Region (CFR), including the province of the Eastern Cape of South Africa (EC) are indicated on the map, namely; the North West (NW), Roggeveld (RV), South West (SW), Agulhas plain (AP), Langeberg (LB), South East (SE) and Karoo Mountain (KM).

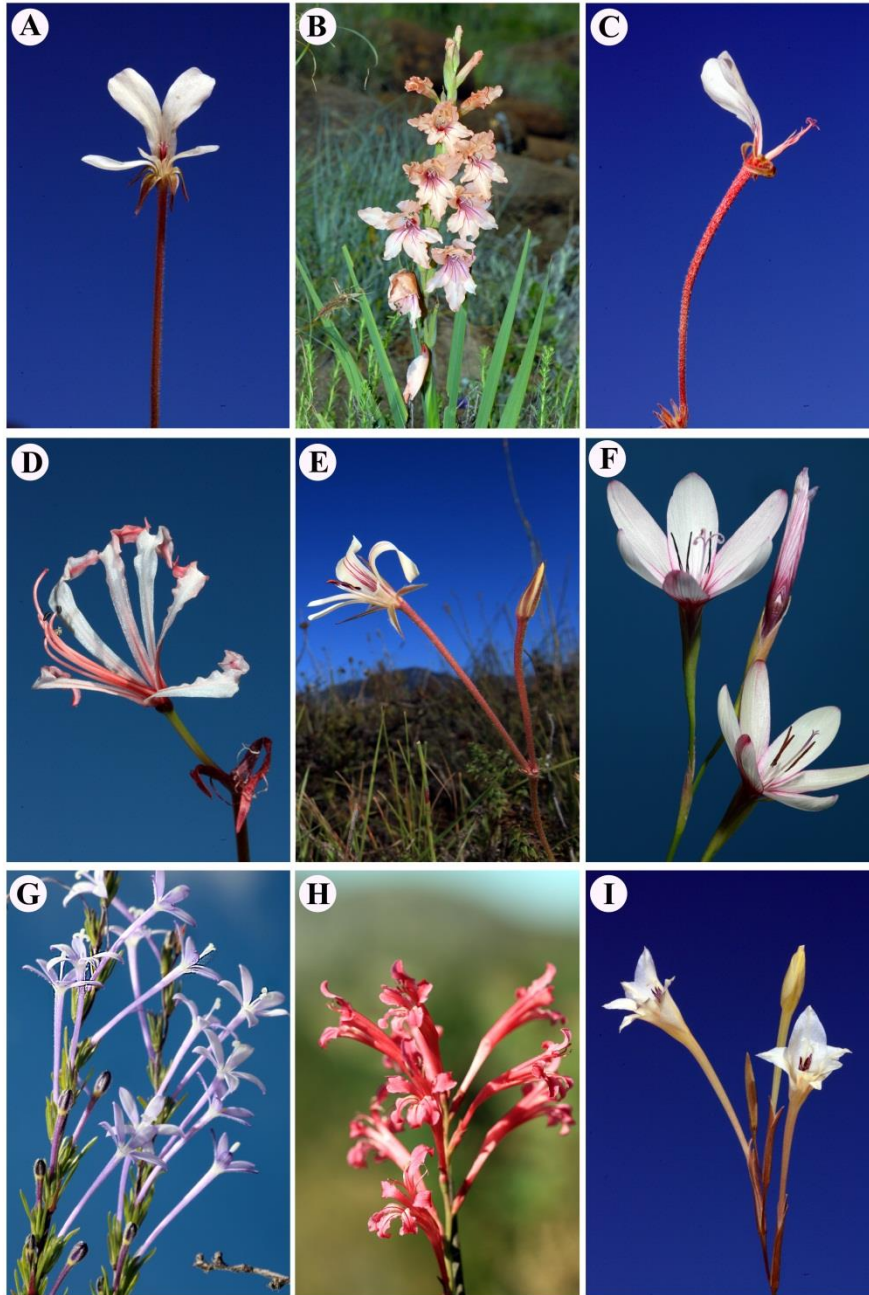


Figure 2.3. A subset of floral guild members pollinated by *P. longipennis*, namely; (a) *Pelargonium pinnatum* (Geraniaceae) (b) *Gladiolus oppositiflorus* (Iridaceae). Photo: Petra Wester (c) *Pelargonium dipetalum* (Geraniaceae), (d), *Nerine humilis* (Amaryllidaceae) (e), *Pelargonium carneum* (Geraniaceae) (f) *Geissorhiza fourcadei* (Iridaceae), (g) *Wahlenbergia guthrie* (Campanulaceae) (h) *Tritoniopsis antholyza* (Iridaceae), and (I) *Gladiolus engysiphon* (Iridaceae).

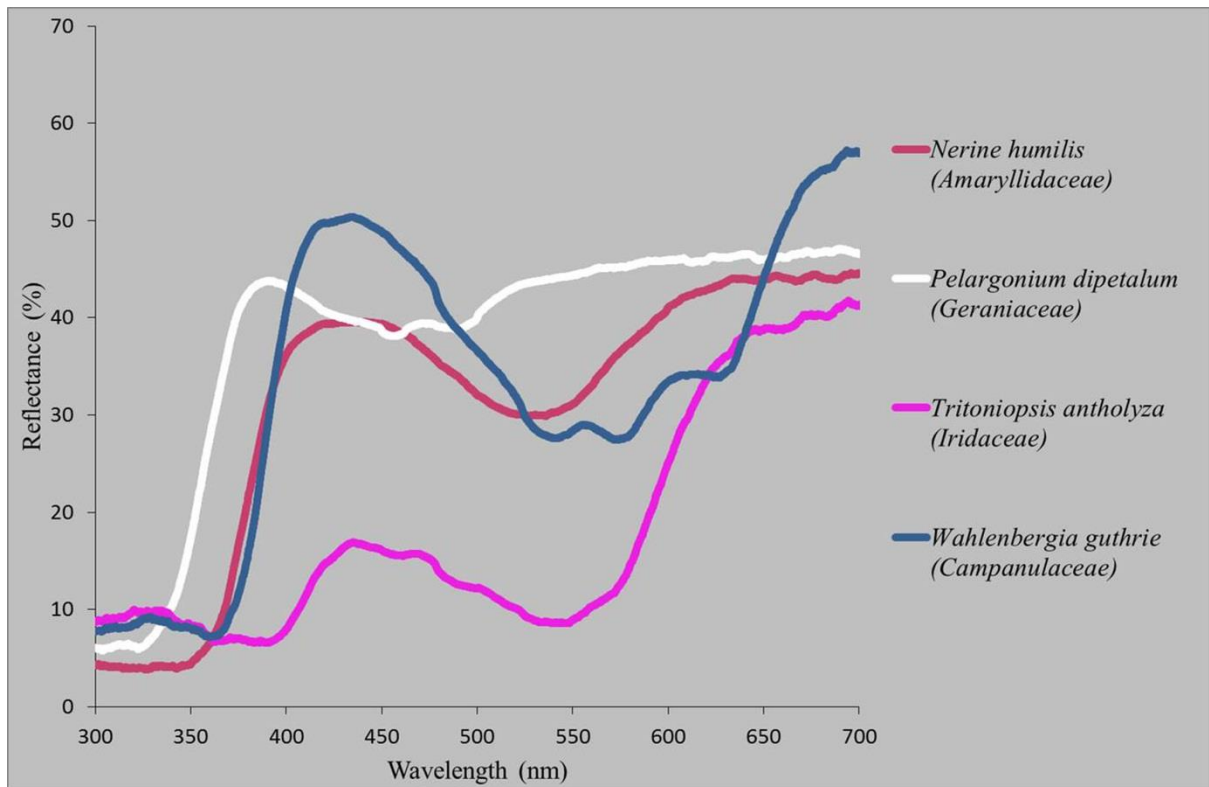


Figure. 2.4. A representative sample of reflectance spectra measured from plants within the *P. longipennis* pollination guild. The different colours on the graph are approximations of colours as perceived by the human eye.

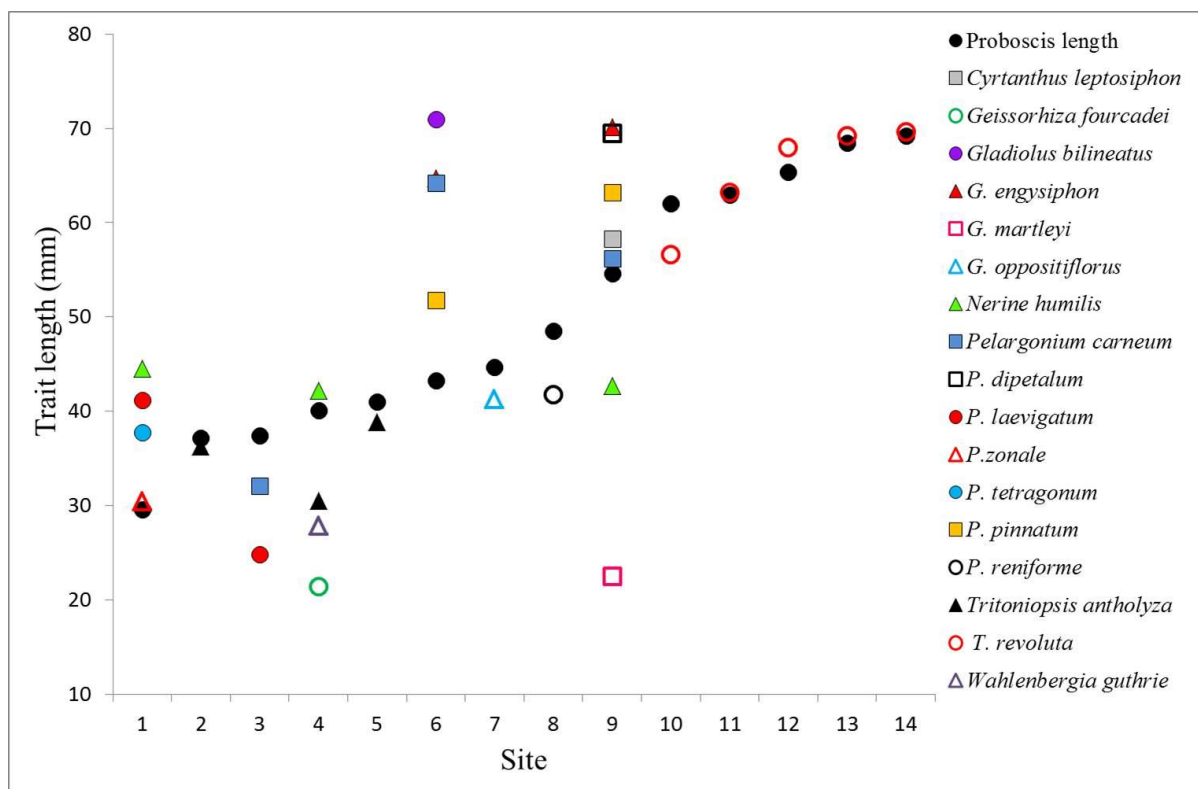


Figure. 2.5. Study sites ranked in order of increasing *P. longipennis* proboscis length, showing the variation in the floral traits associated with proboscis length for each plant species within and among study sites.

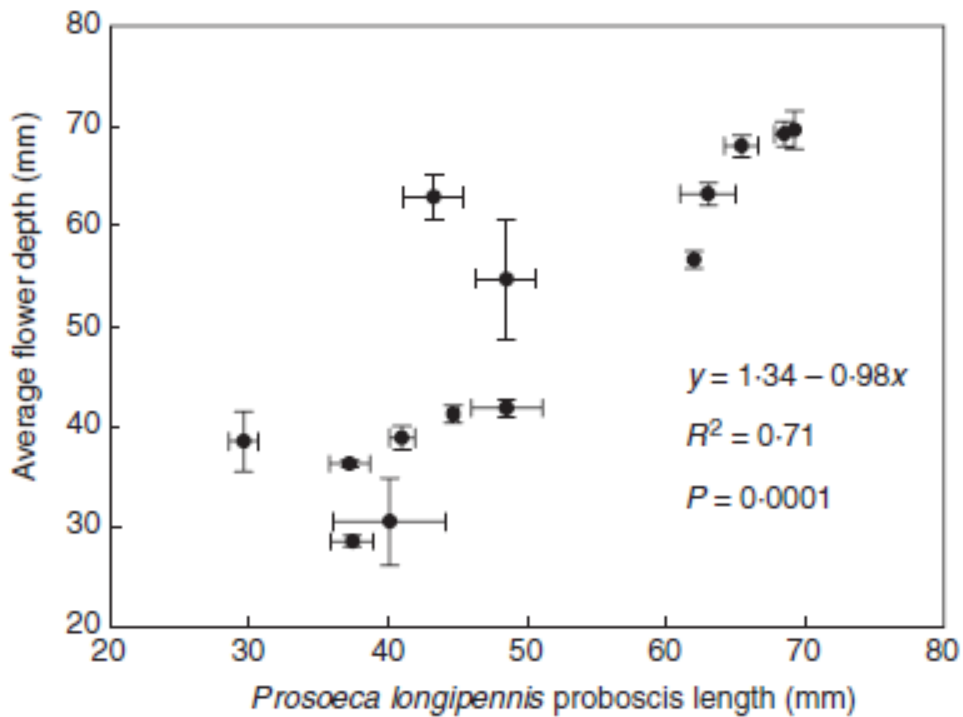


Figure. 2.6. Scatterplot with an ordinary least squares regression, indicating the relationship between the grand means of all guild members per study site and *P. longipennis* proboscis length across those study sites. Each symbol indicates the mean trait value and SE per population.

Chapter 3

LOCAL ADAPTATION: MECHANICAL FIT BETWEEN FLORAL ECOTYPES OF *NERINE HUMILIS* (AMARYLLIDACEAE) AND POLLINATOR COMMUNITIES

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ABSTRACT

Geographic variation in floral morphology is often assumed to reflect geographic variation in pollinator communities and associated divergence in selective pressures. We studied populations of *Nerine humilis* (Amaryllidaceae) to assess whether geographic variation in floral form is the result of local adaptation to different pollinator communities. We first tested for associations between floral traits and visitor communities, and found that populations with similar floral morphologies were visited by similar insect communities. Mean style length in each population was also closely associated with the mean body length of the local visitor community. A reciprocal translocation experiment demonstrated that native phenotypes set more seed than translocated phenotypes. Single visitation experiments showed that native flowers received more pollen, and set more seed per visit, than introduced phenotypes in both populations. This suggests that the effectiveness of pollinator visits is determined by the degree of mechanical fit between flowers and visitors. We provide strong evidence that the observed among-population variation in floral traits is an adaptive response to geographic variation in the pollinator community.

INTRODUCTION

Pollinators select on floral traits through their behavioral preferences or via the mechanical fit between pollinator and floral morphology (Anderson *et al.*, 2010a; Nattero *et al.*, 2010; Van der Niet *et al.*, 2014a). Geographic mosaics in the composition of pollinator communities can generate geographically divergent selective pressures among plant populations (Stebbins, 1970). Divergent selection on floral morphology is expected to lead to morphologically distinct pollination ecotypes, and may potentially drive speciation events (Van der Niet *et al.* 2014a). This is sometimes referred to as the Grant-Stebbins model of pollinator-driven divergence (Johnson, 2006; Johnson, 2010). Phylogenetic studies showing associations between pollinator shifts and speciation events suggest that pollinators may have driven up to 25% of speciation events in animal-pollinated angiosperms (Kay & Sargent, 2009; van der Niet & Johnson, 2012). However, studies of associations between divergence events and pollinators are seldom informative about the actual processes that drove the initial stages of divergence (Nosil, 2012). To bridge this gap between macro-evolutionary patterns and the processes that drove them, it is important to study selection and adaptation in plant populations that are in the process of diverging (Sobel & Streisfeld, 2014). Our approach in this manuscript is to determine firstly whether divergent floral morphology among plant populations is associated with variation in the local pollinator fauna, a pattern that is suggestive of local adaptation to geographically variable pollinators. We then attempt to determine the mechanisms behind the identified patterns through reciprocal translocations between populations.

We investigate local adaptation in populations of the South African geophyte *Nerine humilis* (Jacq.) Herb. (Amaryllidaceae) across a large part of its range. *Nerine humilis* is part of a pollination guild which includes 17 other plant species that are visited by the long-

proboscis fly *Prosoeca longipennis*. *Prosoeca longipennis* has a patchy distribution range in Southern Africa (Newman *et al.*, 2014), and nine of the 17 plant species (including *N. humilis*) appear to display shifts to other pollinator species in populations outside the range of the fly (Newman *et al.*, 2014). *Nerine humilis* exhibits significant geographic variation in the length of stamens and styles. In particular, Newman *et al.* (2014) documented populations with either short styles (grand mean \pm SE: 27.44 \pm 0.74mm, n= 4 populations) or much longer styles (grand mean \pm SE: 43.14 \pm 0.53mm, n = 3 populations). They hypothesized that these floral differences represented adaptive responses to differences in pollinator assembly (i.e. pollination ecotypes), and that geographic differences in pollinator preference or morphology drove the divergence of floral traits. This hypothesis forms the core of the manuscript. We test the hypothesis by investigating patterns of association between floral phenotype and visitor fauna, and present data from experimental approaches aimed at determining the mechanisms behind those patterns.

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

If pollinator mosaics drive the divergence of floral morphology, then we can expect that populations with similar floral morphology should share similar pollinators. Furthermore, the pollinator traits that drive floral divergence (e.g. proboscis length or color preference) should coincide with the floral traits that are presumed to be under selection in each population (e.g. tube length or floral color). Several studies have described geographic patterns of association between plant and pollinator morphology, indicative of floral adaptations to local differences among pollinator communities (Boberg *et al.*, 2014; Johnson *et al.*, 2014; Newman *et al.*, 2014; Sobel & Streisfeld, 2014; Van der Niet *et al.*, 2014b). This pattern-based approach has often revealed very close trait-matching between plants and

pollinators (Anderson *et al.* 2010b), supporting the idea that floral morphology converges when populations are pollinated by similar pollinators, and that it diverges when populations are pollinated by different pollinators. Correlative patterns of association are a useful starting point in the study of floral trait divergence as they are able to demonstrate that trait matching is replicated across multiple populations. While falsification of the trait-matching expectation will reject the hypothesis that pollinator mosaics drive floral diversification, correlative studies are not definitive evidence for the hypothesis because they are limited by the inability to distinguish cause and effect, making it necessary to study the mechanisms behind those patterns (Nuismer *et al.*, 2010).

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

Reciprocal translocations are useful for identifying divergent patterns of selection and for demonstrating local adaptation of putative ecotypes (reviewed in Kawecki and Ebert 2004), and some pollination studies have utilized them to demonstrate that local floral forms have greater female fitness than introduced floral forms (Robertson & Wyatt, 1990; Streisfeld & Kohn, 2007; Sun *et al.*, 2014). However, a limitation of many reciprocal translocation studies is that while they may suggest differing selection gradients on floral traits among populations, it is often difficult to distinguish the agents of selection (Van der Niet *et al.*, 2014a). To overcome this problem, reciprocal translocation studies can also be used to examine the effects of floral phenotypes on specific sub-components of fitness (e.g. visitation rates and pollen deposition) which are unlikely to be affected by anything other than pollinators. As well as identifying pollinators as the agents of selection, this directly links patterns of local adaptation to traits that enhance attraction (e.g. color or scent, *see* Newman *et al.* 2012) or traits that affect the degree of morphological or behavioral fit between flowers and their pollinator selection agents (e.g. (Aigner, 2001; Nattero *et al.*, 2010)). Whenever plants are

introduced into novel populations, the use of seed set as a measure of female fitness may be confounded if incompatibilities exist between native and introduced phenotypes, making it essential to undertake preliminary studies on compatibility between the phenotypes used in reciprocal translocations. Furthermore, both pollen deposition and seed set may be influenced by the position of pollen placement in the host population. In such instances, introduced phenotypes may be pollen limited because plants in the host population deposit pollen on a different part of the pollinator and not because their morphology does not fit with the pollinators' morphology. Consequently, assessing whether pollinators make contact or not with the reproductive parts of flowers can be useful in determining whether reduced seed set or pollen deposition of introduced phenotypes are the results of maladaptation to the local pollinator fauna. Although well-designed reciprocal translocation experiments can be very informative about local adaptation and even about the agents of selection, they are labor-intensive and are thus normally limited to just a few populations. Consequently, it is useful to combine pattern-based approaches which infer local adaptation in multiple populations, with reciprocal translocations that are able to demonstrate it in selected study populations.

We divide this manuscript into two sections that are repeated throughout, and that reflect these two different approaches commonly used to study local adaptation: In the first section we describe patterns of association between pollinators and floral morphology. We predict that if pollinator mosaics have generated floral divergence, then 1) populations with similar visitor assemblages will share similar floral morphology; and 2) that style length in each population should match the functional body length of floral visitors in each population. In the second section, we identify the mechanisms behind the described patterns. An association between pollinator and plant traits may arise if local pollinator preferences or morphology differ among populations and also select for divergent traits. If so, then 3) local

phenotypes should perform better than morphologically different introduced phenotypes. Furthermore, 4) if local adaptation is due to differences in the preferences of pollinators, then we expect local pollinators to visit local phenotypes more frequently than introduced phenotypes. Finally, 5) if local adaptation is due to differences in the fit between plant and pollinator, then we predict that pollen transfer between local plants and pollinators will be more effective than that between introduced plants and pollinators. In particular, the stigmas of local (but not introduced) flowers are expected to make frequent contact with floral visitors. The mechanical fit between pollinator and flower should affect pollen receipt and seed set so that local forms receive more pollen and produce more seeds than introduced forms.

METHODS

Study species

Nerine humilis is a self-incompatible geophyte (Newman, unpublished data) from Southern Africa that flowers in the wild from late March to early June. The umbellate inflorescences carry from one to eight pale to deep pink, zygomorphic flowers lacking any fragrance discernible to the human nose. The flowers lack a nectar tube and nectar is secreted from septal nectaries in the inferior ovary at the base of the polypetalous perianth. Nectar is easily accessible to most floral visitors because a long proboscis is not required to reach it (Fig. 3.1). Six anthers and a single stigma are borne at the ends of slender filaments and style respectively, both of which display significant variation in length across the geographic range of the species (Goldblatt & Manning, 2000a). Flowers of *N. humilis* are protandrous, with distinct male and female phases, and the flowers last between three and seven days each. The male phase is initiated by dehiscence of the anthers, and terminated by recurving of the filaments. Following the male phase, the style elongates and the stigmatic surface becomes

receptive, occupying the former position of the stamens. *Nerine humilis* is relatively widespread through the Cape Floristic Region with a range stretching across $\pm 700\text{km}$ (Goldblatt & Manning, 2000a).

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

Visitor observations

We recorded the abundance and identity of all visitors to *N. humilis* flowers in 11 populations across the Western and Eastern Cape of South Africa (Table S3.1), from March towards the end of May. Visitor observations in each population were conducted for ten hours across two days, between 09h00 and 14h00 on days when ambient temperatures were at least 20° C. For each population, five hours of observation were completed in 2012 and another five hours in 2015. In each population, observations were conducted on approximately 300 flowers located within a radius of two meters, so that a single observer was able to survey the entire demarcated patch without moving.

Measurements of floral morphological traits

Four floral traits (tepal length, nectar concentration, nectar volume and functional style length) were measured for 12 to 45 flowers at each study locality using one mature flower per arbitrarily selected plant. Table 3.1 details the exact replication for the measurement of each trait in each population. Trait measurements were standardized by using only mature flowers in the female phase, recognized by their strongly trilobed stigmas. Tepal length, a surrogate for floral size, was determined as the straight line distance between the base and apex of the dorsal tepal, using digital calipers for 19 to 27 flowers per population (Table 3.1). Nectar concentration and volume were measured in 12 to 37 flowers from each

locality (Table 3.1). Measurements were performed early in the morning before visitors started foraging, and before evaporation could potentially alter nectar readings. Measurements were taken using a 1–5 μl graduated micropipette (Drummond Scientific Company, Broomall, PA, USA) and a 0–50 % Bellingham–Stanley refractometer (Bellingham and Stanley, Tunbridge Wells, UK). Functional style length was recorded from 19 to 45 flowers in each of the 11 *N. humilis* populations (Table 3.1). Functional style length was determined as the distance from the nectary to the tip of the stigma (*see* S3.1 – Measurements of functional floral and visitor traits). The abdomens of floral visitors routinely make contact with the stigmas (Fig. 3.1A), making functional style length a likely trait to be selected on by visitor morphology. Floral color does not generally differ among most populations in this study, but flowers from the SK population used in reciprocal translocations were unusually pale. Consequently, in the two reciprocal translocation populations (SK and NKP), we used a spectrometer (Dunedin, Florida) to measure the dominant tepal color (principal attractive surface) across the range 300–700 nm. These measurements were averaged over five individual flowers from SK and seven individual flowers from NKP.

Measurements of visitor morphological traits

In all 11 populations, functional visitor length was calculated as the combined length of body plus proboscis. We considered this length to be the pollinator trait to which functional style length may be adapted. Functional visitor lengths were measured with digital calipers from a total of 133 insects (Table S3.2) which had been killed using Potassium cyanide. Functional visitor length was determined by the angle at which insects held their proboscides while foraging (*see* S3.1 – Measurements of functional floral and visitor traits).

Is there an association between floral phenotype and visitor assemblage?

We generated a preliminary Bray-Curtis similarity matrix, which has no *a priori* assumptions about grouping, to identify *N. humilis* populations that were associated by their similarities in visitor fauna. The Bray-Curtis similarity matrix was based on the similarities of visitor communities among all 11 *N. humilis* population pairs (Table S3.1) and the Bray-Curtis metric accommodates the relative abundance of different visitors at each site and not just their presence or absence. The matrix was used to plot a cluster analysis which grouped populations in the form of a neighbor joining tree. This neighbor joining tree identified two distinct clusters of populations based on their visitor assemblages; one group comprising eight populations (Group 1) and another of three populations (Group 2) (Fig. S3.1). We tested for a significant difference between the visitor faunas of the two groups using a one way ANOSIM. Significance was estimated by comparing the calculated global *R* value with randomly generated *R* values (10000 permutations) (Clarke & Warwick, 2001). A SIMPER analysis determined that the presence or absence of long-proboscid flies was the most important factor in generating these two groups. Results were visualized using a non-metric multidimensional scaling plot (NMDS). PRIMER 5 version 5.2.9 (Primer-E, ltd) (Clarke & Warwick, 2001) was used for all analyses above.

To test whether similarities in visitor fauna were associated with similarities in floral morphology, we determined whether floral traits (nectar volume, nectar concentration, tepal length, and style length – *see* Table 3.1) differed significantly between the two groups identified by the preliminary analysis (i.e. populations with and without long-proboscid flies). Following a Bray-Curtis similarity matrix on these floral traits, significant differences in the floral traits of these two groups were determined using a one way ANOSIM as above. We also performed a SIMPER analysis to determine which floral traits were the most important associates with the two insect visitor groups.

We also tested for a correlation between the Bray-Curtis differences in floral morphology and visitor assemblage between each pair of populations. Associations between these two matrices were tested using a Mantel test in Excel stat (2014) version 2014.6 package of Microsoft Excel. Our expectation was that if populations are locally adapted to different floral visitor communities, then the matrices should be correlated (i.e. populations with similar floral traits should share similar visitor communities).

To determine specifically which traits differed and how these traits differed between populations with and without long-proboscid flies, we used a Linear Mixed Model (LMM) with phenotype as a fixed factor and locality as a random factor, with locality nested within phenotype. The traits examined were tepal length, nectar concentration, nectar volume, and style length.

Do floral and visitor morphology correlate across populations?

Here we focused on a single floral trait (functional style length) and a single pollinator trait (functional visitor length) to determine the existence of morphological correlations across the 11 populations. Mean functional length of each visitor species was weighted by multiplying its abundance in each population (Table S3.1) with its mean functional length for that population (Table S3.2) to obtain a weighted mean for each visitor species in each population. The weighted means of visitors were summed for each population and divided by the total number of visitations observed for all insects within each population to provide a weighted grand mean of functional visitor length in each population (Table S3.2). Standard errors of the weighted means were calculated using the methods of (Gatz & Smith, 1995). The relationship between mean functional style length and mean functional visitor length was analyzed using a univariate regression with functional style length as the dependent variable, and the grand mean functional visitor length as the predictor variable. For this analysis of

pattern, all floral visitors were included, even those that did not regularly make contact with the reproductive parts of the flowers. We did this because we were interested in whether floral morphology was reflective of the potential selective agents in each population, thereby avoiding the circularity of using only visitors that made frequent contact with the reproductive parts of flowers.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

Are floral phenotypes locally adapted?

To experimentally investigate whether plant populations with short and long styles are adapted to their local environments, we conducted reciprocal translocation experiments between one long-style and one short-style population. The short-style population was located at Skurwekop (SK) near Napier in Western Cape and the long-style population was at Nuwekloof Pass (NKP) in the Baviaanskloof Mountains in Eastern Cape, approximately 380 km distant (*see* Fig. 3.2). Eighteen cut inflorescences with flowers in bud were translocated from SK to NKP, and fifteen similar inflorescences were translocated from NKP to SK. Translocating inflorescences did not affect their ability to set seed (*see* S3.2 - Effects of cut stems and travel on seed set). In each reciprocal translocation population, cut inflorescences were arranged in pairs comprising one native and one introduced inflorescence and placed in water-filled test tubes mounted on skewer sticks, providing a total of 33 inflorescence pairs across the two reciprocal translocation localities.

At each reciprocal translocation locality, inflorescence pairs were arranged at the same height and had the same number of flowers (1 to 4). Within replicated pairs, native and introduced inflorescences were arranged approximately 30 cm apart. For the purpose of recording observations for choice experiments (see below), pairs were arranged approximately 2 m apart in a half circle in front of the observer. Buds were allowed to open

in the field and water was changed every three days. Anthers of all experimental plants in the study were removed while flowers were in bud to prevent contamination of the local gene pool. This process of anther removal had little effect on visitor positioning because the visitors of these flowers were not observed to actively harvest pollen. Furthermore, the anthers usually dehisce and abscise by the time the mature stigma occupies the position formerly held by the anthers. As *N. humilis* is self-incompatible (Newman, unpublished data), all seeds produced by experimental plants were the result of cross-pollination. Inflorescence pairs were left in the field until they showed signs of withering. At this stage they were taken back to the laboratory for fruits to develop, where the proportion of fertilized ovules out of the total number of ovules was determined. Ovule development continued normally in water (S3.2 - Effects of cut stems and travel on seed set) and fertilized ovules were clearly distinguished from aborted ovules by their green and swollen appearance ($> 3\text{mm}$ diameter). In contrast, unfertilized ovules fail to develop beyond small, transparent spheres $\pm 1\text{ mm}$ diameter. These differences were readily determined with the naked eye (Fig. S3.2). Furthermore, eighteen flowers from SK and fifteen flowers from NKP had similar numbers of ovules ($t = 0.86$, $P = 0.66$).

We used generalized estimating equations (GEE's) (Liang & Zeger, 1986) to determine whether native inflorescences produced a higher proportion of fertilized ovules than introduced inflorescences. For each flower, the proportion of fertilized ovules (dependent variable) from each fruit formed per flower was calculated using the total number of events (fertilized ovules) occurring in a set of trials (total number of ovules). Since each inflorescence had several flowers, pair number was used as a repeated subject variable and an exchangeable correlation matrix was used to account for possible correlations of increased visitations to inflorescence pairs with larger displays. (Galen & Newport, 1987). We used a

binomial distribution with a logit link function with factors, source locality and phenotype treated as interaction terms in the model (source locality x phenotype). Furthermore, we also performed pairwise contrasts on the interaction term (source x phenotype) using Sequential Sidak statistics on the estimated marginal means. Hand crosses between plants from SK and NKP showed that the two phenotypes were fully cross compatible and consequently incompatibilities do not confound reciprocal translocation results (S3 – Testing for inter-phenotype incompatibilities).

Are floral phenotypes locally adapted to visitor preferences (Sensory fit)?

The previous experiment was capable of demonstrating local adaptation but it could not identify which traits are being selected upon, or what the agents of selection are. To determine whether differences in seed set are due to traits that vary in their attractiveness to visitors (e.g. color or size), we recorded visitation rates to local and introduced inflorescences within the same experimental pairs as above, with a total of 65 visits at NKP, and 70 visits at SK. We excluded no floral visitors from the analyses. The total number of visits for long and short style phenotypes within inflorescence pairs (replicates) was recorded for three days at each reciprocal translocation locality between 09:00 and 14:00. At the end of each day, the positions of the introduced and local individuals within each pair were swapped around. Total visits to pair members were analyzed using Generalized estimating equations (GEE) with a Poisson distribution and a log-link function, with factors source locality x phenotype treated as interaction terms. The model used an exchangeable correlation matrix to account for non-independence of pollinator foraging on inflorescences. Significance was assessed using generalized score statistics.

Are floral phenotypes locally adapted to visitor morphology (mechanical fit)?

To investigate whether plants are locally adapted to fit mechanically with local visitor morphology, we performed single visitation experiments for a single day in each reciprocal translocation population. This experiment used different flowers from the previous experiments. Flowers were bagged in each reciprocal translocation population, and as soon as stigmas were receptive, inflorescences with a single receptive flower were cut and offered to visitors. Developing anthers were removed from all flowers in bud, thus preventing gene pool contamination. Virgin flowers from each phenotype were offered to honey bees where short-style plants were native, or to long-proboscid flies where long-style plants were native. In the short-style population (SK), we offered 33 short-style flowers and 12 long-style flowers to bees. In the long-style population (NKP), we offered 31 short-style flowers and 14 long-style flowers to long-proboscid flies (*Prosoeca longipennis*). We solicited single visitations by placing flowers in the natural *N. humilis* populations in water filled test tubes, mounted on skewer sticks. The flowers were monitored continuously and each flower was permitted only a single visitation, at which time we recorded whether floral visitors made contact with the mature stigmas of the flowers or not. After a single visit, the inflorescence was placed in the shade and bagged to prevent further visitation. Visited flowers were labelled and taken back to the laboratory where pollen grains were counted on each stigma under a dissecting microscope. Stigmas were not cut or stained as we did not want to compromise seed set. After pollen counting, the inflorescences were placed in regularly changed water until fruits were formed.

We analyzed “contact” versus “no contact” by visitors to native and introduced flowers using a generalized linear model with a binomial distribution and a logit-link function, with source locality x phenotype as interaction terms in the model. Pollen counts from single visitation experiments were analyzed using a Generalized Linear Model (GLM) with a Poisson distribution and a log-link function, with source locality x phenotype as the

interaction term in the model. The proportion of fertilized ovules from single visitations was analyzed using a Generalized Linear Model (GLM) with a binomial distribution and a logit link function. Source locality x phenotype were treated as interaction terms. Pairwise contrasts on the interaction terms of all single visitation analyses described above were investigated using Sequential Sidak and LSD (Least Significant Difference) statistics.

For graphic representation of all models depicted above, we used back transformed values of the adjusted marginal means, which resulted in asymmetric standard errors. See Table S3 for details.

RESULTS

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

A total of 537 observations of 18 insect species were recorded across 11 populations of *Nerine humilis* (Table S3.1). Long-proboscid flies, *Prosoeca longipennis* and *P. ganglebauri* (Nemestrinidae) visited all long-style populations (9, 10, and 11) and were the most abundant visitors in each of these populations. Long-style populations were also visited at lower frequencies by short-proboscid insects (Table S3.1). Long-proboscid flies were never observed at the short-style populations. Fourteen species of short-proboscid insects visited the short-style populations (1-8), with honey bees (*Apis mellifera*) the only species seen at all short-style populations.

Is there an association between floral phenotype and visitor assemblage?

Spatial clustering suggested that the two groups of plant populations identified in the UPGMA analysis differed significantly in their visitor assemblages (ANOSIM: Fig. 3.3A; $R = 0.88$, $P = 0.006$). Honey bees were responsible for 59.33 ± 1.04 (SD) % of the similarity

among the eight short-style populations whereas the long-proboscid fly *P. longipennis* was responsible for 74.18 ± 0.89 (SD) % of the similarity among the three long-style populations.

The type of visitor community (i.e. with or without long-proboscid pollinators) was associated with specific sets of floral traits (ANOSIM: Fig. 3.3B; $R = 0.95$, $P = 0.006$). The most important of these traits was style length, which accounted for 39 ± 12.51 (SD) % of the similarity among populations without long-proboscid flies, and 44.6 ± 34.14 (SD) % of the similarity among populations with long-proboscid pollinators (SIMPER analysis).

Populations without long-proboscid pollinators also clustered through similarities in tepal length (34.4 ± 15.31 % of similarity) and nectar volume (24.93 ± 5.92 % of similarity). Similarly, populations visited by long-proboscid flies also clustered by larger tepals (31.55 ± 23.57 % of similarity) and higher nectar volumes (21.69 ± 4.02 % of similarity). Furthermore, increasing similarity in visitor assemblages between population pairs was correlated with greater similarity in floral morphology (tepal length, nectar volume, nectar concentration, style length) between populations (Fig. S3.3; Mantel $r = 0.66$, $P < 0.001$).

Populations with long-proboscid pollinators had significantly longer styles ($F_{1, 8.64} = 109.51$, $P < 0.001$), longer tepals ($F_{1, 8.76} = 11.09$, $P = 0.009$), and larger nectar volumes ($F_{1, 9.2} = 33.08$, $P < 0.001$) than populations without long-proboscid pollinators. There was no significant difference in nectar concentrations among populations with and without long-proboscid fly pollinators (Table 3.1; $F_{1, 8.71} = 3.92$, $P = 0.080$). Although tepal color did not appear to differ among populations with and without long-proboscid flies, clear color differences between the two populations used in the choice experiment could be seen with the human eye and with the aid of a spectrophotometer (Fig. S3.4).

Do floral and visitor morphology correlate across populations?

We observed a strong match between functional style length and functional visitor length across populations (Fig. 3.4; $R^2 = 0.75$, $P = 0.001$). Populations with short style lengths were associated with visitors that had short functional body lengths, and populations with long style lengths were associated with visitors that had longer functional body lengths.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

Are floral phenotypes locally adapted?

A significant source locality x phenotype interaction (Table 3.2, Fig. 3.5; $X^2 = 39.40$, $P < 0.001$) in the reciprocal translocation experiments provides strong evidence for local adaptation (Kawecki & Ebert, 2004). All pairwise contrasts for this experiment were significant (Table 3.2; $P < 0.02$), suggesting that both long- and short-style phenotypes are locally adapted.

Are floral phenotypes locally adapted to visitor preferences (sensory fit)?

A total of 65 choices were observed at NKP (59 from *P. longipennis*, 3 from *Prosoeca willowmorensis*, and 3 from *Apis mellifera*) and 70 choices at SK (61 from *Apis mellifera*, 6 from *Lasioglossum sp.*, and single choices from *Eristalinus tenax*, *Anthene definita* and *Amegilla spilostoma*). Although there appeared to be a pattern of local adaptation in which local phenotypes received more visits than introduced phenotypes, this pattern was not significant (Table 3.2, Fig. 3.6A; interaction term $X^2 = 5.47$, $P = 0.141$).

Are floral phenotypes locally adapted to visitor morphology (mechanical fit)?

Single visitation experiments suggest that a greater proportion of floral visitors makes contact with the stigmas of the native phenotypes than with introduced phenotypes (interaction term: Fig. 3.6B; $X^2 = 19.70$, $P < 0.001$). All contrasts were significant (Table 3.2;

$P < 0.025$). This translated into local phenotypes receiving more pollen grains per visit than introduced phenotypes (interaction term: Fig. 6C; $X^2 = 1697.00$, $P < 0.001$). All contrasts were significant (Table 3.2; $P < 0.001$). Differences in pollen deposition also translated into differences in seed set (interaction term: Fig. 3.6D; $X^2 = 120.90$; $P = 0.001$), with all contrasts significant (Table 3.2; $P < 0.001$).

DISCUSSION

Several lines of correlative evidence suggest that floral traits in populations of *Nerine humilis* visited by long-proboscid flies differ from those in populations not visited by long-proboscid flies. Furthermore, experimental evidence suggests that geographic differences in pollinator morphology drive local adaptation of floral traits through the mechanical fit between floral and pollinator morphology. Theoretically, divergence in floral traits can arise through genetic drift, phenotypic plasticity, or natural selection (Herrera *et al.*, 2006). Taken together, our evidence suggests that the floral traits of *N. humilis* populations have diverged as a result of natural selection imposed by different pollinator communities. Below, we discuss the hypothesized links between floral morphology, visitor composition, and visitor morphology as correlative support for pollinator-driven trait divergence. We then discuss experimental evidence for local adaptation of *N. humilis* populations to different kinds of pollinators, addressing the mechanisms behind the observed patterns.

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

Nerine humilis populations across the range of the species are visited by two distinct visitor communities, and the type of visitor community is predicted by the floral morphology of each population. Populations with long styles, higher volumes of nectar, and larger

flowers are visited predominantly by long-proboscid flies, while populations with short styles, less nectar, and smaller flowers are visited only by functionally smaller pollinators. Moreover, we show that the greater the morphological similarity between any pair of populations, the greater the similarity in the visitor fauna. Of the floral traits under study, we focused on the trait most closely associated with the functional size of the visiting insect, viz. style length. We found a strong positive association between average style length of *N. humilis* flowers and the weighted grand mean of visitor functional body length in each population. This type of geographic trait matching among pollinator-plant populations has been found in other studies (Steiner & Whitehead, 1990a; Anderson & Johnson, 2008; Pauw *et al.*, 2009; Newman *et al.*, 2014) and appears to be a general trend among specialist plant species and their pollinators (Anderson *et al.*, 2010b; Armbruster *et al.*, 2014). Geographic associations between plant morphology and pollinator species, as well as trait matching at the population level, are both expected outcomes of local adaptation in plant-pollination interactions (Van der Niet *et al.*, 2014a). They provide supportive evidence that plants are locally adapted to phenotypic differences in their visitors at each site, or that visitors are locally adapted to plants at each site (Boberg *et al.*, 2014; Sun *et al.*, 2014). Alternatively, both plant and visitor traits may be phenotypically plastic and similarly affected by an external factor such as temperature or altitude (Strauss & Whittall, 2006). Although we have not demonstrated a genetic basis to the trait differences that we identified, several published studies show heritability in floral tube length (Campbell, 1996; Worley & Barret, 2000), a trait which is frequently correlated with style length. In addition, we observed that considerable inter-population variation in style length in *N. humilis* is maintained in the bulb collections of growers, suggesting a heritable component to this trait.

Despite multiple possible sources of variation in floral morphology, floral form has frequently been used to make predictions about pollinator identity, and associations between

the two have been used to infer pollinator-driven evolution (Valente *et al.*, 2012). A prominent example are pollination syndromes, defined as suites of floral traits associated with particular pollinators (Johnson, 2010). Thus, sunbird pollinated plants often have unscented, red, tubular flowers, plenty of nectar, and a structure for birds to perch on while foraging (Anderson *et al.*, 2005a), whereas moth pollinated flowers are often recognized by pale flowers with long tubes and nocturnal fragrance (Johnson, 2010). Although the concept of pollination syndromes is pervasive in the pollination literature, their predictive power remains controversial among some authors (Rosas-Guerrero *et al.*, 2014). Here we show that syndromes may extend to the level of populations in *N. humilis*, and that floral morphology still has persuasive predictive power about the identity of important floral visitors even at the population level. Nevertheless, patterns of association on their own do not actually show that there is, or has been, divergent selection by pollinators acting on floral traits. Nor are correlative patterns able to determine whether plants are adapting to local pollinator traits or *vice versa* (Nuismer *et al.*, 2010). For this we used experimental approaches to elucidate the mechanisms behind the patterns of association.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

We demonstrated at both sites that local phenotypes set more seed than introduced phenotypes, suggesting that populations of *N. humilis* are locally adapted to each site. Through mechanical fit experiments we showed that plants are in fact locally adapted to floral visitors, thereby enabling us to identify them as the agents of selection. In preference experiments, the site-by-phenotype interaction was not significant, despite the appearance of a pattern of local adaptation (Fig 3.6A). This suggests either that differences in floral phenotypes are not the result of differences in pollinator choice or that pollinator choice plays a very weak role in driving floral divergence of these two populations. The weakness of the

interaction effect is unlikely to be an artefact of low replication since the replication in this experiment was more than three times greater than any of the other experiments presented here. Instead, it suggests that performance differences in reciprocal translocations may rather be explained by traits relating to other mechanisms such as the mechanical fit between pollinators and flowers (c.f. Fig. 3.5 and Fig. 3.6D).

We found long-proboscid flies to be much more effective visitors of the long-style phenotype, making frequent good contact with the stigmas of the flowers (Fig. 3.1A, Fig. 3.6B), resulting in good pollen deposition (Fig. 3.6C) and high seed set (Fig. 3.6D). In contrast, long-proboscid flies seldom made contact with the stigmas of short-style flowers (Fig. 3.1B, Fig. 3.6B), seldom deposited pollen on these stigmas (Fig. 3.6C), and their visits resulted in a lower proportion of fertilized ovules (Fig. 3.6D). The opposite was true of honeybees, which frequently made contact with short-style flowers (Fig. 3.1D, Fig. 3.6B) but not long-style flowers (Fig. 3.1C, Fig. 3.6B), resulting in good pollen deposition (Fig. 3.6C) and a higher proportion of fertilized ovules in short-style flowers than in long-style flowers (Fig. 3.6D). This suggests that the proportion of fertilized ovules produced may be strongly influenced by the morphological match between flower and pollinator. Since anthers are positioned similarly to stigmas in *N. humilis*, the “lock-and-key-fit” is also likely to affect components of male fitness such as pollen export. Consequently, aspects of male fitness may also select on floral morphology such as the length of the anther filaments and their reciprocal correspondence with style length. Contact data demonstrated that performance differences in pollen receipt and seed set are not just the result of introduced phenotypes being in a population in which pollen is placed on some other part of the pollinator body. Instead, performance differences are the result of introduced phenotypes having a poor morphological fit with the local pollinators.

An alternative pollinator-driven explanation for variation in floral morphology is that floral traits evolve to exclude less efficient pollinators (Johnson *et al.*, 2006) rather than being adaptations to a particular pollinators. Tube length is a case in point: it may have evolved either to exclude inefficient pollinators (Borrel, 2005) or as an adaptation to the morphology of a specific pollinator (*sensu* (Darwin, 1862)). In reality it is often difficult to disentangle these differences. Style length variation in *N. humilis* is an unusually informative trait to study because its origins are less ambiguous. Style length does not inhibit access of pollinators to nectar, and so variation in this trait is most likely to influence only the efficacy of pollen transfer and not the likelihood that different pollinators will visit. Because long styles (unlike long nectar tubes) do not restrict access to nectar, style length is unlikely to select for longer proboscis lengths, whereas tube length may (e.g. Pauw *et al* 2009). Nevertheless, our data suggest that the functional length of insects may be an important selective force on style length in *N. humilis*. Thus style variation among populations appears to represent adaptation of plants to pollinators and not *vice versa*. In a similar way, the spurs of nectarless orchids appear to track the coevolutionary races between rewarding plants and their pollinators, without selecting on the pollinators themselves (Anderson *et al.*, 2005b; Anderson & Johnson, 2009; Anderson *et al.*, 2010b; Thompson, 2013). This so called divergent evolution (Anderson *et al.*, 2005b) is in contrast to coevolution where both pollinators and plants adapt to one another, as has been proposed for many long-tubed plant systems (Darwin, 1862; Muchhala & Thomson, 2009; Pauw *et al.*, 2009)

Our findings lend support to the pattern-based approaches of macroevolutionary studies which so frequently associate shifts in pollinators with plant speciation (van der Niet & Johnson, 2012). Most importantly, we illustrate how floral morphology affects the efficiency of pollen transfer, and that the presence of morphologically different visitors selects for differences in floral morphology. This positively identifies pollinators as agents of

floral selection in *N. humilis*, and demonstrates some of the mechanisms behind morphological trait matching in pollinators and plants. We show that adaptations to pollinators can lead to geographic divergence in floral traits, and that different forms of *N. humilis* can be considered as pollination ecotypes (Van der Niet *et al.*, 2014a), viz. local forms that have diverged as a result of pollinator differences.

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Table 3.1: Short and long-style *N. humilis* study localities, with population means, standard errors and (sample sizes) for floral traits. Statistical differences between trait magnitudes of short and long-style phenotypes from the linear mixed model (LMM) are given in bold. Populations 4 and 9 represent reciprocal translocation localities SK and NKP.

Statistics	Population	Phenotype	Style length	Tepal length	Nectar volume	Nectar concentration	Functional Visitor length	Latitude	Longitude
	1	Short	21.56±0.91 (20)	20.80±0.94 (20)	0.67±0.17 (20)	23.86±2.48 (18)	15.16	33.54	20.60
	2	Short	23.42±0.89 (21)	18.87±0.89 (21)	0.56±0.16 (21)	15.19±2.29 (21)	16.83±0.67	34.07	20.39
	3	Short	24.09±0.78 (27)	20.65±0.78 (27)	0.75±0.10 (24)	17.85±1.73 (37)	18.11±1.61	33.70	19.07
	4	Short	24.44±0.61 (45)	21.61±0.61 (20)	0.42±0.74 (31)	10.80±2.35 (20)	15.86±1.06	34.46	19.91
	5	Short	25.20±0.85 (23)	21.46±0.85 (23)	0.76±0.18 (18)	15.00±2.48 (18)	14.11±0.32	34.48	19.92
	6	Short	27.44±0.70 (34)	29.42±0.70 (19)	0.83±0.17 (20)	17.89±2.48 (18)	15.33±0.71	34.19	20.29
	7	Short	28.46±0.83 (24)	25.60±0.83 (24)	0.70±0.07 (21)	17.58±3.04 (12)	18.56±1.97	34.41	19.77
	8	Short	29.24±0.93 (19)	24.10±0.93 (19)	0.87±0.16 (23)	25.52±2.30 (21)	15.78±5.23	34.44	19.74
	9	Long	41.23±0.87 (22)	29.92±0.74 (22)	3.86±0.14 (27)	16.96±2.10 (25)	31.49±6.36	33.51	23.64
	10	Long	42.15±0.74 (30)	29.92±0.74 (26)	2.36±0.18 (18)	33.38±2.63 (16)	37.56±0.08	33.39	22.56
	11	Long	42.22±0.81 (25)	29.10±0.81 (25)	1.85±0.15 (24)	27.06±1.83 (33)	54.78±1.80	34.02	20.60

Grand Means (short)	-	25.48±2.66	22.81±3.39	0.70±0.05	17.96±4.78	16.22±0.54	-	-
Grand Means (long)	-	41.87±0.55	29.65±0.47	2.69±1.04	25.80±8.28	41.28±6.98	-	-
<i>P</i> (LMM)	-	< 0.001	<0.001	<0.001	<0.080	-	-	-
Total sample size	-	290	246	247	239	133	-	-

Table 3.2: Pairwise contrasts on the interaction terms of local adaptation experiments, and similar experiments investigating mechanisms underlying local adaptation, significant values are indicated in bold.

Test	Experiment	Experimental localities	Phenotype	Locality*phenotype (X^2)	Locality*phenotype (P)	Contrasts within source locality (P)	Contrasts between source localities (P)
Local adaptation	Reciprocal translocation	NKP	Long			<0.001	<0.001
		SK	Short	39.40	0.001	0.019	0.002
Sensory fit	Preference experiment	NKP	Long			-	-
		SK	Short	5.47	0.141	-	-
Mechanical fit	Single visitations (contact)	NKP	Long			0.004	0.001
		SK	Short	19.70	<0.001	0.006	0.024
Mechanical fit	Single visitations (pollen deposition)	NKP	Long			<0.001	<0.001
		SK	Short	1697.00	<0.001	<0.001	<0.001
Mechanical fit	Single visitations (seed set)	NKP	Long			<0.001	<0.001
		SK	Short	120.90	<0.001	<0.001	<0.001

Table S3.1. Observation data, documenting all visitors recorded from short (S) and long-style (L) localities 1 -11. Numbers represent the total visitors observed to flowers during observation periods. Reciprocal translocation localities are highlighted in bold.

Insect species	Locality										
	^s 1	^s 2	^s 3	^s 4	^s 5	^s 6	^s 7	^s 8	^L 9	^L 10	^L 11
<i>Prosoeca</i> sp.	0	0	0	0	0	1	0	0	0	0	0
<i>Prosoeca</i> sp. 2	0	0	0	0	0	0	0	0	6	0	1
<i>Prosoeca longipennis</i>	0	0	0	0	0	0	0	0	21	1	14
<i>Prosoeca ganglebauri</i>	0	0	0	0	0	0	0	0	8	1	0
<i>Apis mellifera</i>	3	62	17	25	7	76	9	2	10	0	5
<i>Chrysomya albiceps</i>	0	1	0	0	7	8	2	0	0	0	0
<i>Chrysomya marginalis</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Anthene</i> sp.	0	0	0	0	0	4	0	0	0	0	0
<i>Anthene definita</i>	1	0	0	6	100	15	2	0	0	0	0
<i>Podalonia canescens</i>	0	0	0	2	5	10	13	0	0	0	0
<i>Amegilla spilostoma</i>	0	0	4	1	2	5	5	1	0	0	0
<i>Xylocopa caffra</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Xylocopa rufitarsus</i>	0	0	0	0	1	1	1	1	0	0	0
<i>Patellapis</i> sp.	6	0	0	6	0	3	0	10	0	0	0
<i>Lasioglossum</i> sp.	0	0	0	4	3	2	3	0	0	0	0
<i>Australoechus hirtus</i>	0	0	0	3	0	0	0	0	0	0	0
<i>Tabanus</i> sp.	2	6	0	0	0	0	0	18	0	0	0
<i>Eristalinus tenax</i>	1	0	1	1	2	0	1	1	0	0	0
Total species observed	5	4	3	8	8	10	8	7	4	2	3

Table S3.2. Means \pm standard errors, and (number) of insect visitors measured to obtain the functional visitor length in (mm) at short (S) and long-style (L) localities used in the non linear regression analysis. Reciprocal translocation localities are highlighted in bold.

Insect species	Locality										
	^s 1	^s 2	^s 3	^s 4	^s 5	^s 6	^s 7	^s 8	^L 9	^L 10	^L 11
<i>Prosoeca sp</i>	-	-	-	-	-	22.53(1)	-	-	-	-	-
<i>Prosoeca sp. 2</i>	-	-	-	-	-	-	-	-	23.84 \pm 0.52(4)	-	-
<i>Prosoeca longipennis</i>	-	-	-	-	-	-	-	-	39.72 \pm 1.24(10)	37.48 \pm 0.92(6)	54.78 \pm 1.81(12)
<i>Prosoeca ganglebauri</i>	-	-	-	-	-	-	-	-	36.49 \pm 0.49(2)	37.64(1)	-
<i>Apis mellifera</i>	15.16 (1)	15.73 \pm 0.67(5)	14.9(1)	16.07 \pm 0.29(9)	15.9 \pm 0.59(3)	14.90 \pm 0.42(5)	15.47 \pm 0.44(4)	9.89(1)	14.83 \pm 0.33(3)	-	-
<i>Chrysomya albiceps</i>	-	-	-	-	11.09(1)	11.56 \pm 0.81(2)	-	-	-	-	-
<i>Chrysomya marginalis</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Anthene sp</i>	-	-	-	-	-	16.55 \pm 0.96(2)	-	-	-	-	-
<i>Anthene definita</i>	-	-	-	-	14.3 \pm 0.42(5)	14.48 \pm 0.36(7)	-	-	-	-	-
<i>Podalonia canescens</i>	-	-	-	20.61 \pm 0.95(6)	-	20.50 \pm 6.61(8)	21.62 \pm 0.54(7)	-	-	-	-
<i>Amegilla spilostoma</i>	-	-	19.18 \pm 0.73(3)	15.93 \pm 0.45(5)	-	17.54 \pm 0.91(9)	16.68 \pm 0.59(2)	21.66(1)	-	-	-
<i>Xylocopa caffra</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Xylocopa rufitarsus</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Patellapis sp.</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Lasioglossum sp.</i>	-	-	-	9.5 \pm 0.46(4)	11(1)	11.87 \pm 1.49(3)	-	-	-	-	-
<i>Australoechus hirtus</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Tabanus sp.</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Eristalinus tenax</i>	-	-	-	-	-	-	-	-	-	-	-
Total species	1	1	2	4	4	8	3	2	4	2	1
Grand Mean	15.16	15.73	17.04\pm2.14	15.53\pm2.28	13.07\pm1.22	16.24\pm1.37	17.92\pm1.88	15.78\pm5.89	28.72\pm5.76	37.56\pm0.08	54.78

Table S3.3. Adjusted means and standard errors from backtransformed values of the linear predictor variable, used in analysis of local adaptation and the underlying mechanisms of local adaptation.

			Backtransformed mean	Upper SE	Lower SE
Fig 5	NKP	Long	0.61	0.68	0.54
		Short	0.18	0.25	0.14
	SK	Long	0.12	0.17	0.08
		Short	0.4	0.46	0.35
Fig 6A	NKP	Long	3.03	3.56	2.59
		Short	2.34	2.66	2.05
	SK	Long	1.66	2.08	1.34
		Short	3.49	4.26	2.86
Fig 6B	NKP	Long	0.78	0.87	0.66
		Short	0.22	0.31	0.16
	SK	Long	0.16	0.19	0.15
		Short	0.58	0.62	0.53
Fig 6C	NKP	Long	33.58	35.16	32.07
		Short	0.48	0.63	0.37
	SK	Long	0.33	0.55	0.2
		Short	29.28	30.33	28.44
Fig 6D	NKP	Long	0.37	0.41	0.33
		Short	0.06	0.08	0.05
	SK	Long	0.03	0.045	0.019
		Short	0.19	0.21	0.18

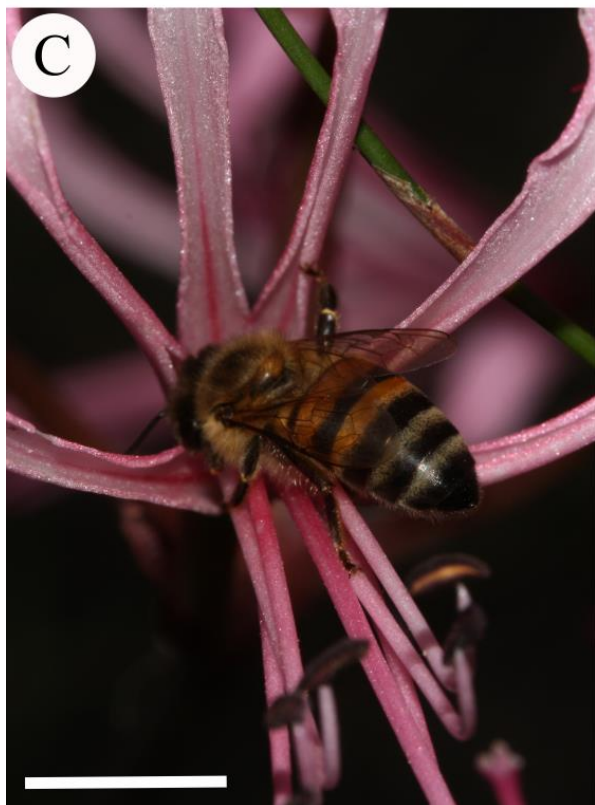
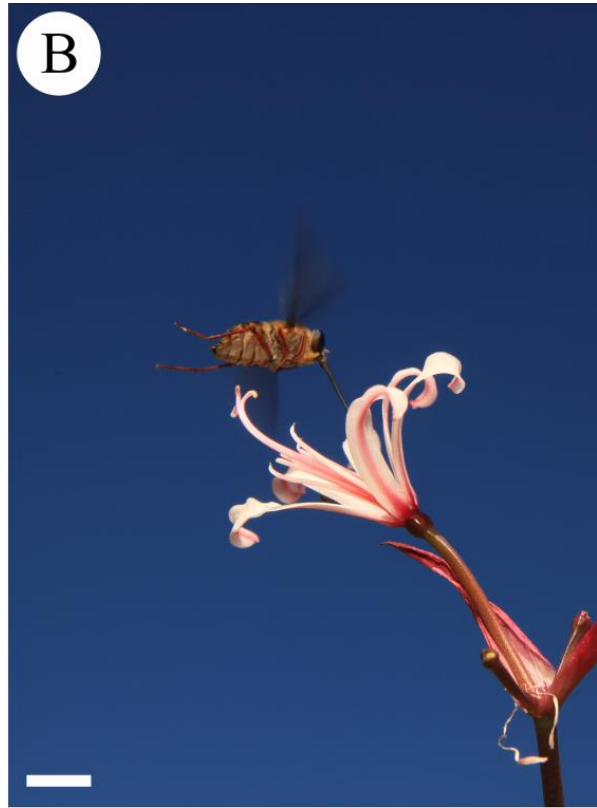


Figure. 3.1. Flowers from the two translocation populations (NKP and SK), being visited by long-proboscid flies and honey bees. (A) The long-proboscid fly *Prosoeca longipennis* visiting the native long-style phenotype (population NKP) of *N. humilis* where the stigma makes contact with the abdomen of the fly. (B) *P. longipennis* visiting an introduced short-style phenotype (from population SK) during a single visitation experiment. Here, the fly is thieving nectar because it makes no contact with the mature stigma. Note that the anthers have been removed. (C) Honeybees, *Apis mellifera* were occasionally observed visiting the native long-style phenotype (population NKP), where they seldom make contact with the reproductive parts of the flowers and pollen was not placed on their abdomens. (D) A honey bee visiting the native short-style phenotype (population SK) of *N. humilis*. Notice pollen deposition on the abdomen of the bee. Scale = 10mm.

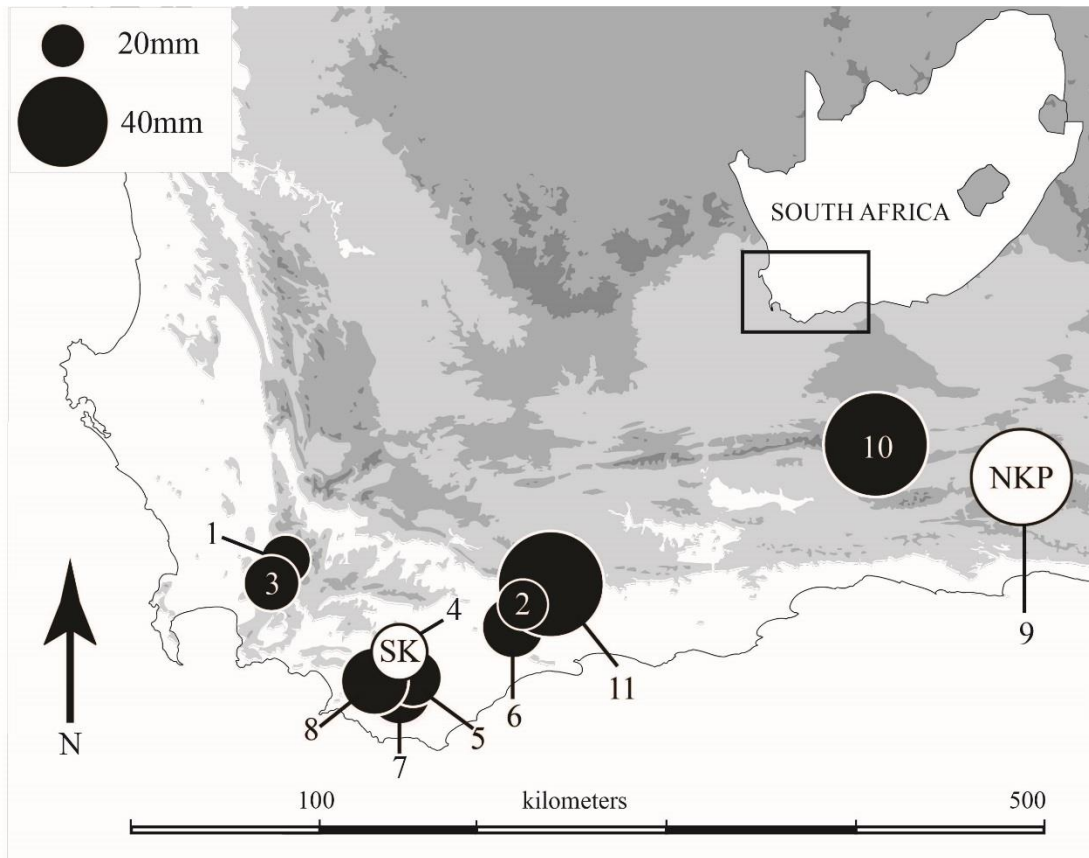


Figure. 3.2. The localities of all study sites used, representing the range of *N. humilis* in the Western and Eastern Cape, South Africa. Circle diameters represent mean style lengths per population. Localities highlighted in white, labeled SK (Skurwekop) and NKP (Nuwekloof Pass) are where translocation studies were performed.

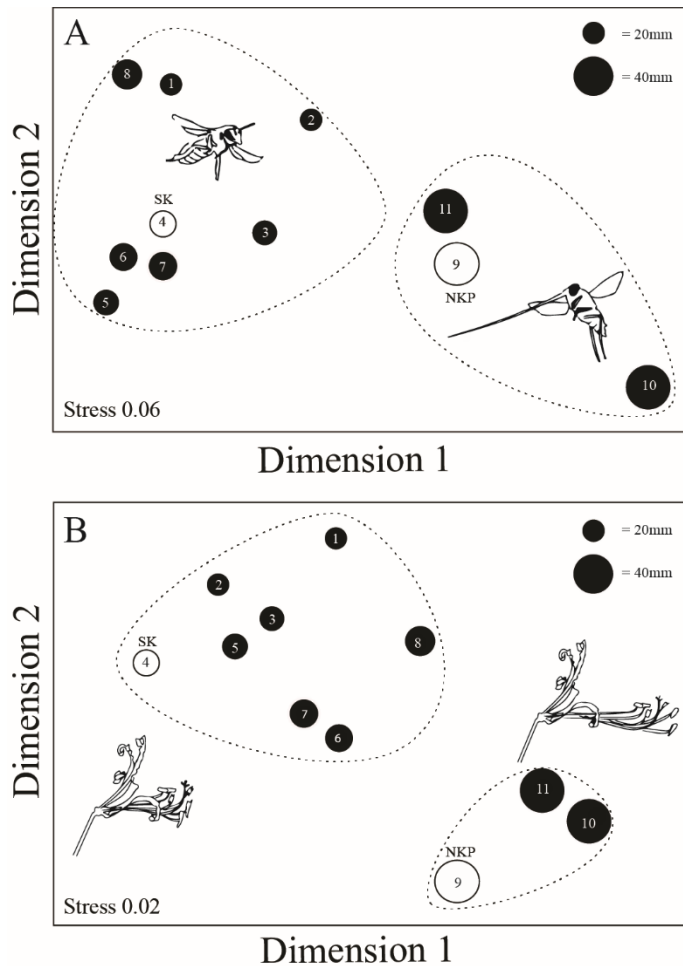


Figure. 3.3. Non-metric multidimensional scaling of associations between visitor community composition and floral morphology. (A) Visitor composition of short and long-style *N. humilis* populations clustered as two significantly different groups in non-metric multidimensional space. Long-style populations cluster primarily as a result of long-proboscid fly visitors and short-style populations cluster primarily due to an abundance of honey bee visitors. (B) Populations with and without long-proboscid flies cluster as two distinct groups on the basis of floral traits. Style length was the primary trait generating the clustering of populations visited by long-proboscid pollinators and populations without long-proboscid pollinators. Circles represent mean style lengths of *N. humilis* populations, while numbers refer to the geographic positions of populations identified in Fig. 2.

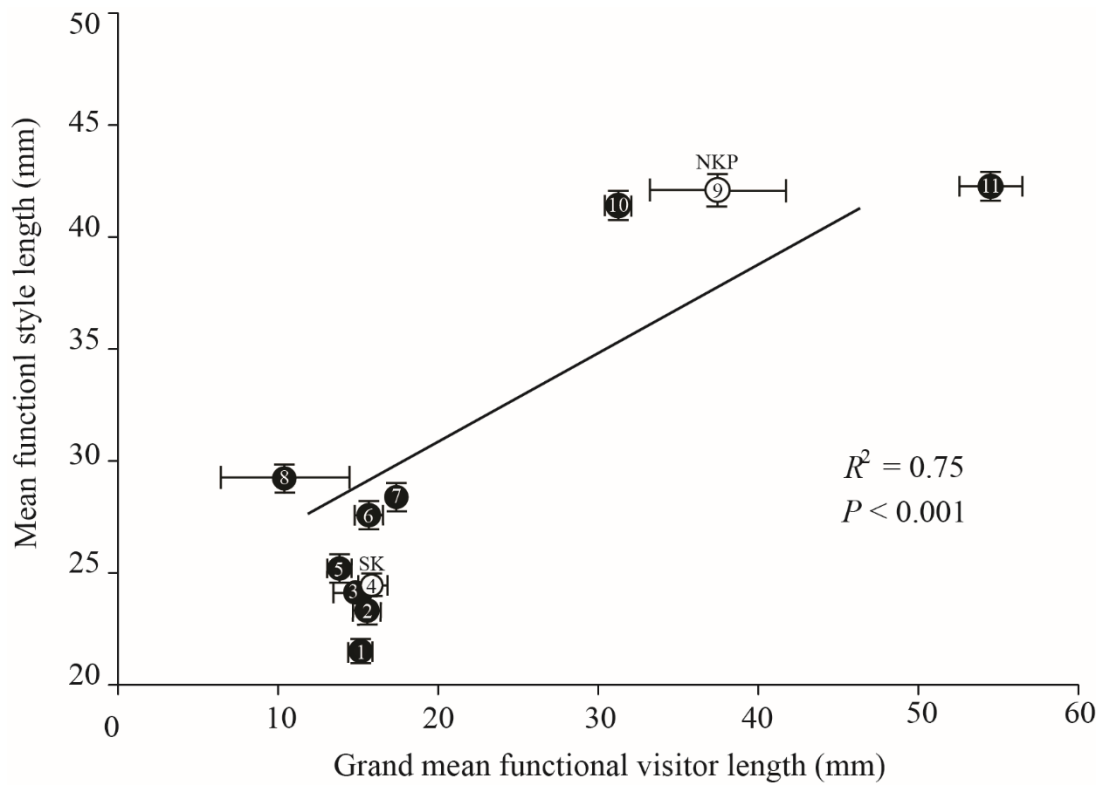


Figure. 3.4. Mean functional style length with standard errors, plotted against grand mean functional visitor length (adjusted to incorporate the relative abundance of different visiting species) with weighted standard errors. Numbers represent study populations and reciprocal translocation localities SK and NKP are indicated by white circles.

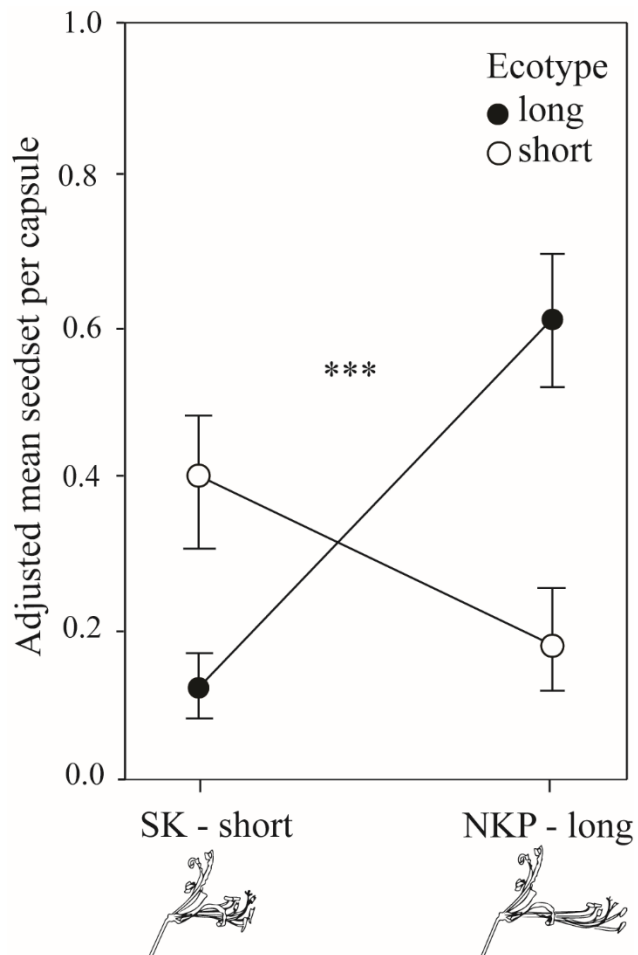


Figure. 3.5. Reciprocal translocations measuring the effects of floral phenotype and locality on female fitness (proportion of fertilized ovules). Here, access to flowers by pollinators was not controlled. A significant interaction between source locality and floral phenotype (short and long-style flowers) indicates that native flowers set a higher proportion of fertilized ovules than introduced flowers. Significant contrasts (Table 2) suggest local adaptation in both populations.

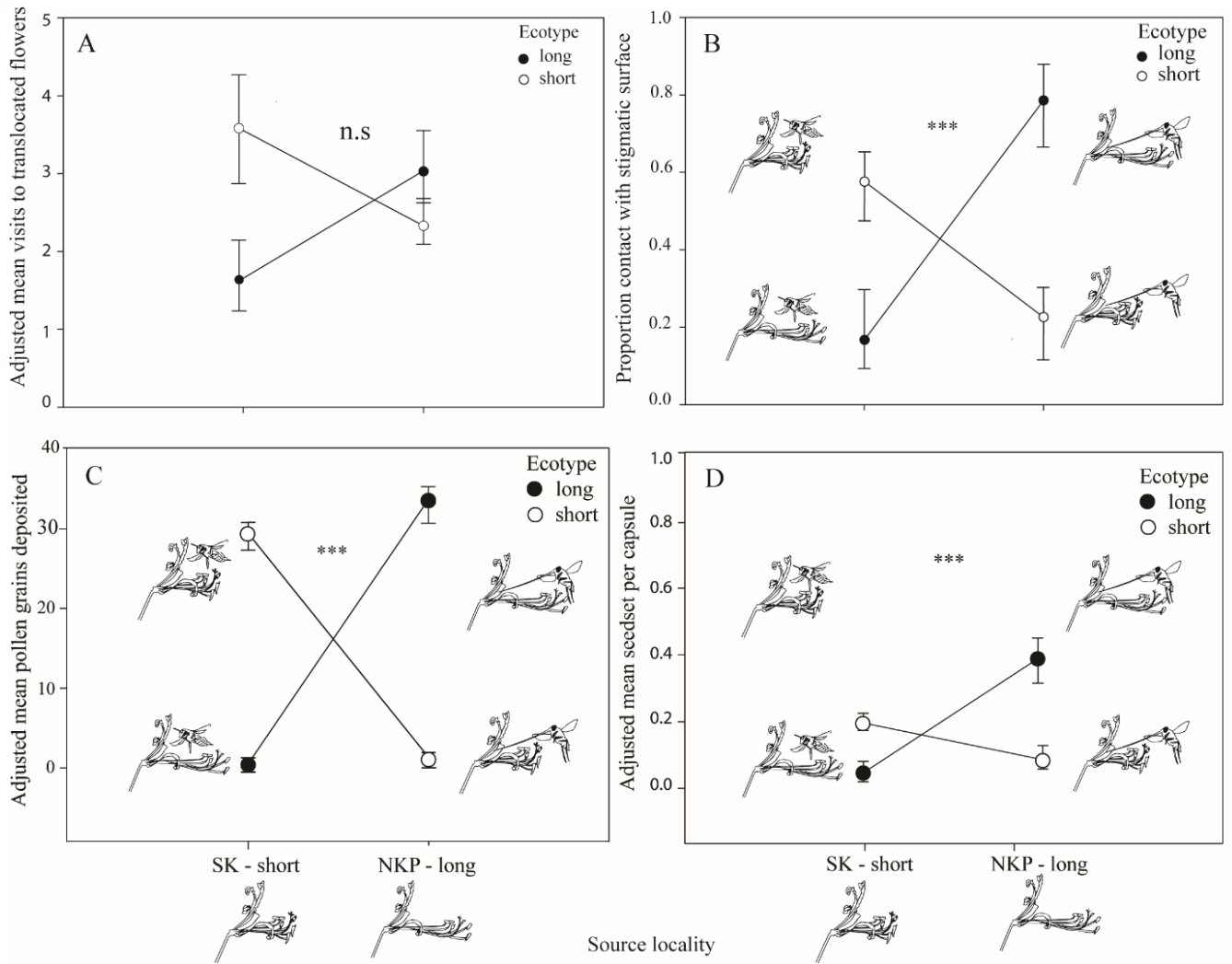


Figure. 3.6. Reciprocal translocations on sub-components of female fitness, designed to distinguish the kinds of traits being selected on, and the agents of selection. (A) A non-significant interaction effect suggests that floral traits involved with pollinator attraction play little or no role in explaining local adaptation. (B) Pollinators had a significantly higher rate of making contact with stigmas of their native phenotypes than introduced phenotypes, suggesting that the mechanical fit between plant and pollinator may affect pollen transfer in both populations. (C) After a single pollinator visit (by bees at SK and by long-proboscid flies at NKP), pollen was consistently deposited more effectively on native phenotypes than introduced phenotypes. This suggests that the identity of the pollinator affects the efficiency of pollen transfer. (D) Differences in the efficiency of pollen transfer translated into differences in the proportion of seeds produced after single visitations by either bees or long-proboscid flies. Diagrams on the X axis represent the style lengths of the experimental source localities. Stars represent significant interactions at $P < 0.01$ *; $P < 0.001$ **; $P < 0.0001$ **

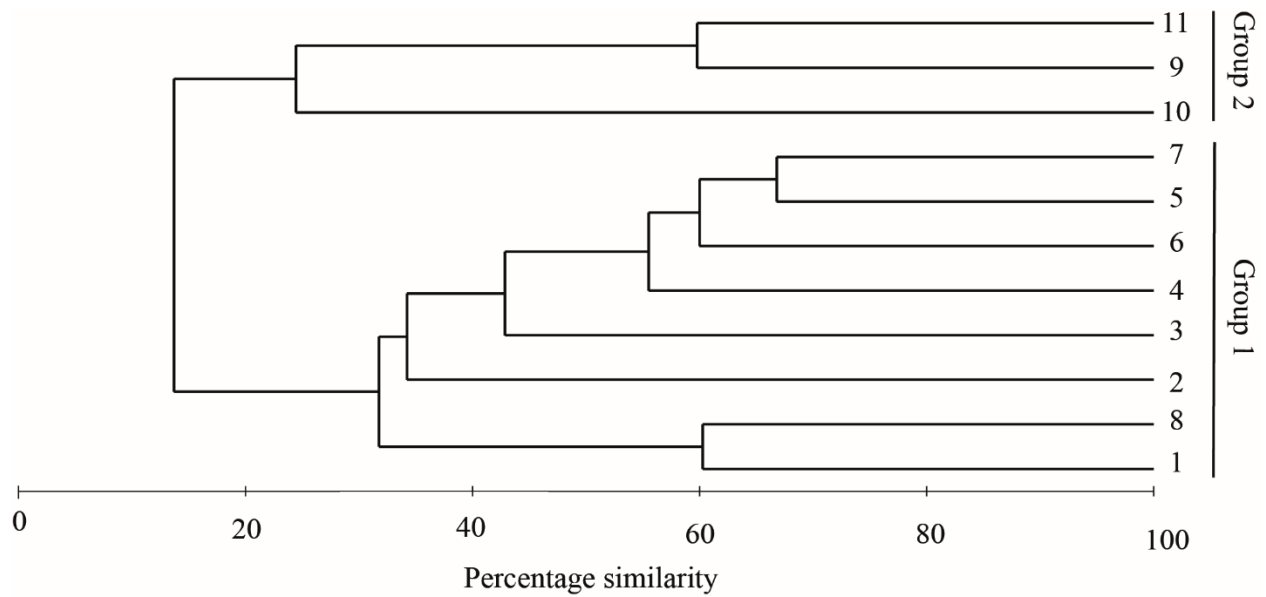


Figure S3.1. Cluster analysis based on the relative abundances of different visitors to each population. Two distinct groups of populations (Group 1 and 2) can be recognised based on similarities of visitors. Populations are labelled from 1-11, and these numbers correspond to the geographic map of the populations (Figure 2).

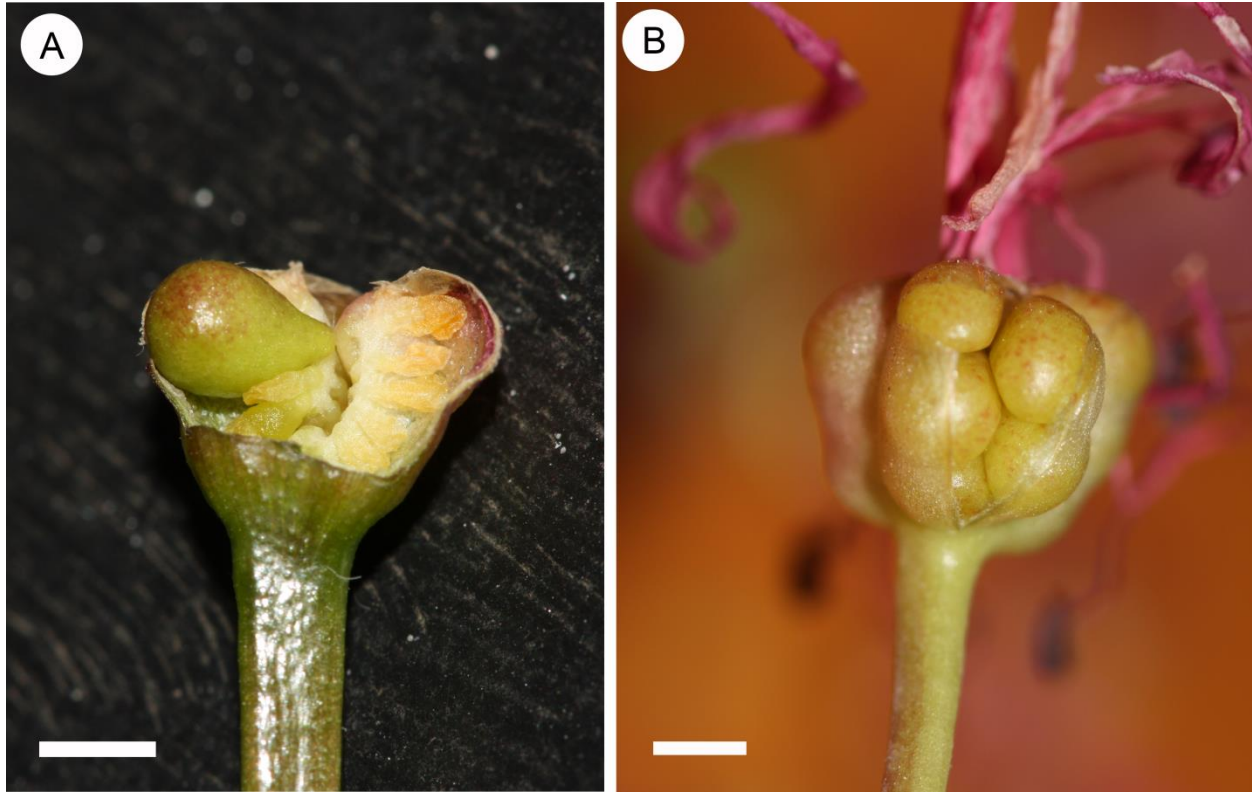


Figure S3.2. Fruits used to determine the proportion fertilized ovules in field and lab experiments. Aborted ovules are characterized by their small (± 1 mm) and shriveled appearance (A), whereas fertilized ovules are characterized by their large and swollen appearance ($\pm > 3$ mm) (B). Scale = 1cm

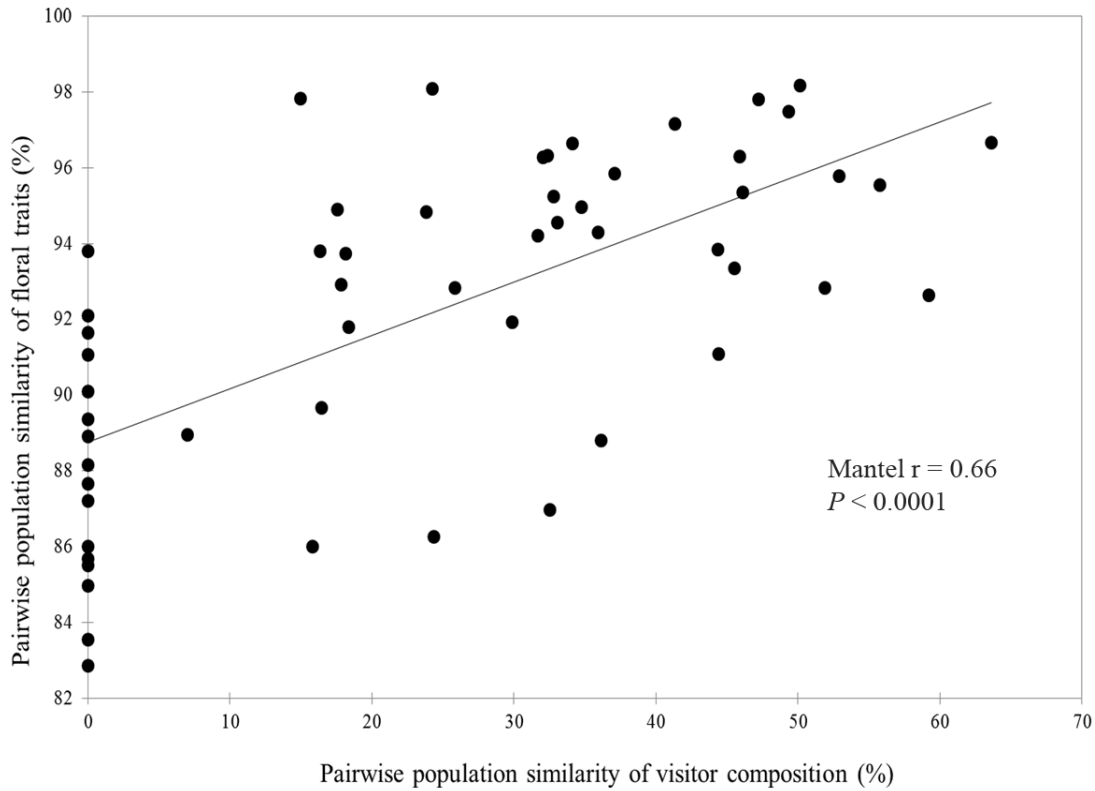


Figure S3.3. Pairwise correlation of population similarity of floral traits (style length, petal length, nectar volume and nectar concentration) and pairwise population similarity of visitor composition used in the Mantel test.

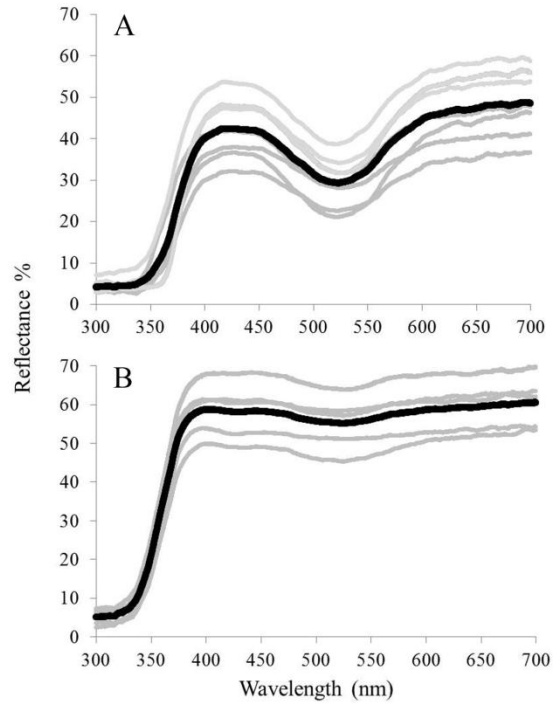
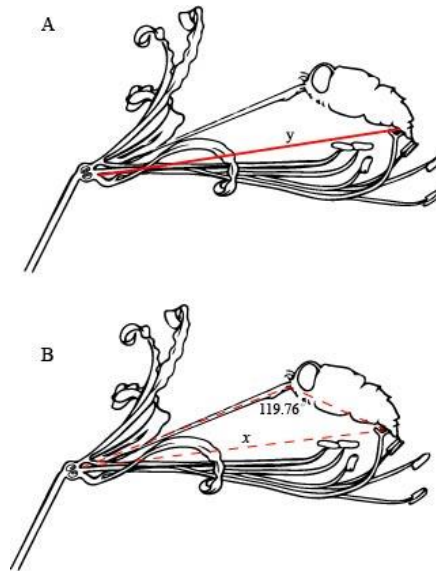


Figure S3.4. Reflectance spectra indicating colour differences of flowers native to reciprocal translocation localities (A) NKP: NuweKloof Pass and (B) SK: Skurwekop. Grey lines represent individual floral measurements, whereas the solid black line represents the mean reflectance.

S1 – Measurements of functional floral and visitor traits



Long-proboscid fly pollinating a long-styled *Nerine humilis* flower, redrawn from a photograph to illustrate (A) Functional style length = y , measured from the base of the nectar chamber to the furthest distance on the stigmatic surface. (B) Functional visitor length = x . x is derived using the cosine formula which requires the proboscis length, body length and “foraging angle.” An average foraging angle (119.76°) was obtained from 5 photographs, which were measured between the proboscis and a line lengthwise through the centre of the flies body.

Insects with short proboscides contacted the stigmatic surface when oriented parallel to the style during foraging. Functional visitor length of all short-proboscid visitors was thus determined as the sum of proboscis length plus body length.

S2 - Effects of cuttings and travel on seed set.

The use of cut inflorescences in reciprocally designed experiments may potentially be confounded by reduced seed set, caused by differences in the amount of resources or disturbance of cut inflorescences versus inflorescences from plants in situ. These difference may further also be increased by additional travelling.

To investigate whether ovule production of cut inflorescences developed similarly to plants in situ. At one site (NKP), we compared the proportion of fertilized ovules of natural seedset from 20 uncut, natural inflorescences to the proportion fertilized ovules of 15 inflorescences placed in test tubes. Both sets of inflorescences were from the NKP site (Fig. 2) and were exposed to the identical pollinator and climatic environment. Differences in the proportion of fertilized ovules produced were analyzed using a generalized linear model (GLM) with a binomial distribution, and logit link function (weighted for overdispersion).

In addition, we incidentally tested for the effects of travelling on the seed production using cut, native Skurwekop (SK) inflorescences used for reciprocal translocations. These inflorescences had travelled double the distance of the introduced inflorescences from NKP. The native inflorescences had travelled from SK to NKP and back to SK. However, introduced plants were only transported once from NKP to SK, travelling only half the distance than plants native to SK. If travelling has a detrimental effect on seed production, we should expect a lower proportion of fertilized ovules from the more well-travelled inflorescences.

Results

The proportion fertilized ovules for cut stems at the NKP locality was the same as the proportion fertilized ovules for untouched stems at the same site ($\chi^2 = 1.240$, $P = 0.265$), suggesting that fruit development continues normally on cut stems held in water. Furthermore, travelling time during translocation also appeared to have no detrimental effect on seed set, since the local experimental inflorescences at SK set more seed than the introduced inflorescences, despite the fact that they had travelled further than the introduced inflorescences (See Fig. 5).

S3 Testing for inter-phenotype incompatibilities

In pollination studies, a problem with many reciprocal translocations conducted between incipient (ecotypes) or closely related species, is that reduced seed set of introduced ecotypes/species may simply be the result of incompatibilities between them (introduced ecotypes/species may experience acute mating limitation if they are incompatible with the local ecotype/species).

Methods

To test whether patterns consistent with local adaptation is simply a consequence of incompatibilities between ecotypes. We performed crosses within and between long and short-styled *N. humilis* reciprocal translocation source localities. Whole bulbs in bud, with at least 3 flowers per inflorescence, were removed from reciprocal translocation localities SK and NKP, and planted individually in 15cm pots using local soil. These were taken back to the field station and placed outdoors in full sun. Budding inflorescences were individually bagged prior to anthesis to prevent pollen contamination from potential pollinators, and once the styles were strongly trilobed and mature, two treatments were performed per inflorescence. Using fine forceps, one flower per inflorescence was pollinated using three pollen donors from individuals within the same population (SK; n = 20, NKP; n = 17), and a different flower on the same inflorescence was cross pollinated using pollen from three different donors from the alternative population (SK; n = 20, NKP; n = 21). The proportion fertilized ovules was determined as soon as it was possible to clearly distinguish fertilized from aborted ovules.

Seed set differences between crosses were analyzed using a Generalized Linear Model (GLM) with a binomial distribution and a logit link function. This model was weighted for

overdispersion and the interaction term between phenotype (long and short-styled populations) and pollen donor used within crosses was investigated. A significant interaction effect between maternal and paternal parents is indicative of incompatibilities between phenotypes. In contrast, a non-significant interaction would suggest that seed set patterns supporting local adaptation could not have been the result of incompatibilities between local and introduced plants. All above statistical analyses were performed in SPSS 20 (IBM; SPSS Inc., Chicago, USA).

Results

Crosses within and between *Nerine humilis* phenotypes were equally inter-fertile. Consequently, there was no significant interaction of seed set between maternal phenotype and the source of the pollen donor ($X^2 = 0.006$; $P = 0.937$, Fig. 1.). In addition, none of the pairwise contrasts were significant. Therefore, seed set results from all reciprocally designed experiments are a consequence of local adaptation, and are not explained by incompatibilities between phenotypes.

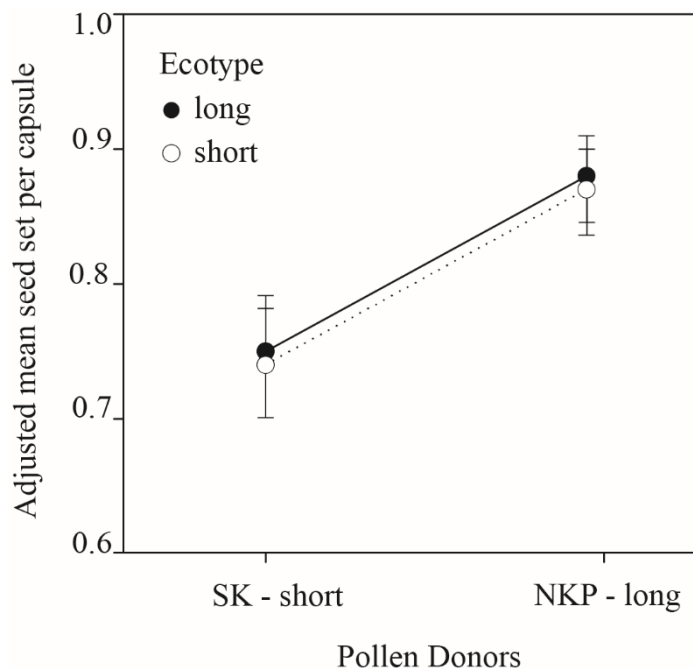


Figure 1. Crosses performed between and within long and short style reciprocal translocation localities, showing no significant interaction in the proportion of fertilized ovules between maternal and paternal parents.

Chapter 4

NATURAL SELECTION FOR MECHANICAL FIT IN POLLINATION ECOTYPES OF TWO SOUTH AFRICAN GEOPHYTES.

ABSTRACT

Strong evidence suggests that pollinator-mediated selection is an important driver of floral divergence and speciation in plants especially when geographically variable selection is imposed by different functional pollinator types. However, few studies have investigated whether pollinator-mediated selection is heterogeneous across landscapes and even fewer have demonstrated that different pollinators select for different floral traits. Here we investigate the strength and form of pollinator-mediated selection on tube and style length in two geophytes that are visited by different functional pollinator types across their ranges. Using single visitations, we demonstrate that functionally different pollinators exert different patterns of selection on floral traits. In contrast, functionally similar pollinators exert similar patterns of selection on floral traits. We also show that functionally similar pollinators from different populations exert similar patterns of selection. Furthermore, populations with more than one functional pollinator type, experience very strong selection by some pollinators and very weak selection by others, leading to fitness surfaces with multiple peaks. When fitness surfaces were broken up into their distinct pollinator components, these fitness peaks matched the morphology of pollinators which exerted strong selection. Our study provides rare evidence of selection on floral traits driven by multiple pollinators within and between populations. Importantly, it challenges researchers to think beyond the simplistic evolutionary models of floral evolution pioneered by Grant and Stebbins.

INTRODUCTION

In allopatric populations, pollinator-mediated selection often plays a pivotal role in driving floral divergence and speciation in plants with specialized pollination systems (Johnson, 2006). In support of this idea, a number of studies have shown local adaptation and consequent divergence of geographic floral forms pollinated by functionally different pollinators (*See van der Niet, et al 2014*). However, surprisingly few studies have investigated natural selection across geographic pollinator mosaics (Agren *et al.*, 2013; Chapurlat *et al.*, 2015). In generalized plant systems, the role of pollinators in driving divergence is less certain because selection by functionally different pollinators may result in conflicting selection. For example, adaptations which are beneficial in the presence of one pollinator may reduce fitness in the presence of a different pollinator (i.e. opposing selection gradients can lead to no net selection on a trait) (Strauss *et al.*, 2005; Sahli & Conner, 2011). This could also lead to uneven fitness surfaces with multiple peaks and valleys which make it difficult for plants to adapt to, or specialize on one particular pollinator (Aigner, 2005; Sahli & Conner, 2011). That said, selection studies on generalist plants have shown divergent selection on floral traits which are dependent on the relative abundances of different functional pollinator types across populations (Gomez *et al.*, 2008). While these selection mosaics on floral traits exist for generalist plants, it is unclear how they are related to the actual pollinators. This is because few studies have broken down selection into components mediated by different pollinators (Castellanos *et al.*, 2004; Gomez *et al.*, 2008; Gomez *et al.*, 2009; Sahli & Conner, 2011; Chapurlat *et al.*, 2015). Aigner's (2001) work was pivotal in demonstrating that we need to consider how the additive effects of all floral visitors affect floral evolution, because the additive effects of multiple visitors can lead to unexpected

fitness peaks. For example, flowers may adapt to less effective pollinators, if adaptations to less effective pollinators do not preclude visits by more effective pollinators.

To break down pollinator driven selection on generalist flowers into some of its components, one can examine how different floral traits affect pollen transfer efficiency after single visits by different kinds of pollinators. Fitness surfaces can then be estimated separately for each pollinator species and composite fitness surfaces can be calculated by merging them after taking the relative abundances of the different functional pollinators into account. However, this is likely to generate complex fitness surfaces, making it difficult to use conventional Lande and Arnold regression based techniques (Lande & Arnold, 1983) to determine selection estimates (selection gradients and differentials).

Cubic splines make no *a priori* assumptions about the fitness surface, allowing researchers to visualize complex fitness surfaces more accurately than by fitting simple directional, stabilizing and disruptive selection curves (Schluter, 1988; Schluter & Nychka, 1994). However, one problem with spline based techniques is that it is often difficult to relate splines to selection gradients which can be compared in a standardized form (Svensson & Calsbeek, 2012) and so, they have primarily been used to illustrate fitness surfaces (Benkman, 2003; Kuchta & Svensson, 2014). However, Morrisey and Sakrejda (2013) have developed a method to extract selection gradients from complex fitness surfaces. This procedure determines selection gradients by averaging the slope (and curvature) of the fitness function (cubic spline) over the entire distribution of values from the observed phenotype, which provides a way to analyze complex selection surfaces. Furthermore, measures of fitness are often measured as survival/death or as counts, which are often highly inflated with scores of zero. Here, the methods devised by Morrisey and Sakrejda (2013) allow researchers to extract selection

gradients from spline surfaces with binomial or poisson error distributions or from data sets which are strongly zero-inflated.

In this study, we extract standardized selection coefficients from complex, pollinator driven selection surfaces of floral traits using cubic splines following the methods developed by Morrisey and Sakrejda (2013). We examine two plant species which are pollinated by long-proboscid flies in some populations and by short proboscid pollinators in other populations. In several previous studies, authors have demonstrated that floral traits such as tube and style length appear to be adaptations to pollinator proboscis length (Anderson & Johnson, 2008; Pauw *et al.*, 2009). When the proboscis lengths of pollinators are geographically variable, tube and style lengths covary in accordance (Anderson & Johnson, 2008; Pauw *et al.*, 2009; Newman *et al.*, 2014; Paudel, 2016). Authors have attributed the covariation between pollinator and intraspecific floral traits to the idea that pollen placement on different parts of a pollinator's body is not equally effective (e.g. Newman *et al.* 2015). For example, in plants pollinated by long-proboscid flies, pollen is usually placed on the body of the fly, because the proboscis of the fly does not offer a large and complex enough surface area for efficient pollen transfer. In *Nerine humilis* (Fig. 1A & B), pollen is placed on the abdomen of insects by exerting stigmas and anthers on the ends of long filaments and styles (Newman *et al.*, 2015). However, in most other taxa pollinated by long-proboscid insects, flowers like those of *T. revoluta* (Fig. 4.1C & D) have evolved long tubes, forcing the pollinators to enter the flower gape (gullet) to access nectar where the body of the pollinator makes contact with the stigmas/anthers.

We use pollen deposition (a component of female fitness) after single pollinator visitations to investigate natural selection on traits that enhance the mechanical fit between flower and pollinator morphology in two *N. humilis* and two *T. revoluta* populations. Because

the number of visits to each flower is standardized, pollen deposition after single visitations is particularly useful in determining how pollen transfer efficiency is affected by variation in floral morphology. The population pairs were deliberately chosen because the plants within those populations differed strongly in their putative mechanical fit morphology and visitor fauna. One *N. humilis* population had short styles and anthers and was only visited by insects with short proboscides, while the other had long style lengths and was almost exclusively visited by insects with long-proboscides. Similarly, one *T. revoluta* population was visited by a combination of bees with short proboscides and flies with medium proboscides while the other was visited by bees with short proboscides and flies with exceptionally long-proboscides. Our primary aims were to contrast patterns of selection by different pollinators within populations and then to contrast patterns of selection across populations with different pollinator compositions

We tested the following hypotheses: 1) we predict that functionally different pollinators will exert contrasting patterns of selection and that 2) morphologically similar pollinators are likely to exert similar selective pressures 3) We predict that populations of the same species with functionally different suites of pollinators should experience divergent selection. 4) If mechanical fit between flower and pollinator morphology is responsible for the shape of the fitness surface, we expect to find matches between the fitness peaks in different populations and the morphology of the most important pollinators. We also expect that the average floral traits in each population should also correspond to calculated fitness peaks.

MATERIALS AND METHODS

Study species and localities

Nerine humilis (Amaryllidaceae) and *Tritoniopsis revoluta* (Iridaceae) are self-incompatible, protandrous geophytes (Ros *et al.*, 2011; Newman *et al.*, 2015) that flower from March-June in the winter rainfall region of South Africa. Both species are pollinated in some populations by the long-proboscid fly *Prosoeca longipennis*, which has a proboscis that can reach over 60mm in length. Both species have evolved pollination ecotypes which are pollinated by short proboscid insects when they occur outside the range of the fly and consequently, floral reproductive parts (e.g. style length in *N. humilis* and tube length in *T. revoluta*) vary geographically in accordance with the morphology of the most effective pollinators (Anderson *et al.*, 2014; Newman *et al.*, 2015). For each species, we studied pollen deposition following single visitations as a component of female fitness in two populations with contrasting phenotypes and pollinators. When multiple pollinator species occurred in a single population, we attempted to examine pollen deposition separately for each pollinator in addition to their combined effects.

Nerine humilis was studied at Nuwekloof pass (-33.511291°S, 23.642786°E) in a population with long styles (style length: mean± SE; 41.23±0.87 mm (n=22)) that match the total functional pollinator length (body plus proboscis lengths) of their long-proboscid fly pollinators *P. longipennis* (mean± SE; 39.72±1.24mm (10)) and *P. ganglebauri* (mean± SE; 36.49±0.49mm (2)) (See Newman *et al* 2015). A population with short styles (style length: mean± SE; 27.44±0.70mm (34)) was also studied at Swellendam (-34.184574°S, 20.291919°E) where anecdotal observations to experimental flowers revealed visitations by short proboscid insects (almost entirely by honey bees) which have total body lengths of (mean± SE; 14.90±0.42 mm

(5)). *Tritoniopsis revoluta* was studied at a long tubed population in Barrydale (-33.935997°S, 20.679453°E), (tube length: mean± SE; 65.86±0.73mm (107)) and a short tubed population in the Swartberg mountains (-33.362594°S, 22.068132°E) (tube length: mean± SE; 25.2±2.15mm (10)) (See Anderson *et al* 2014). Flowers in the Barrydale population are visited by solitary bees with short proboscides (mean± SE, 8.66±0.94mm (4)) as well as *P. longipennis* flies with exceptionally long proboscides (mean± SE; 65.4±1.23mm (7)). The *P. longipennis* flies in this population have longer proboscides than the *P. longipennis* flies where *N. humilis* was studied at Nuwekloof pass. In the Swartberg pass, *T. revoluta* is visited by an unidentified fly with a proboscis of only 23.36±1.12mm (11) (mean± SE) as well as two species of bee, both of which have proboscides which are shorter than 23.36mm in length.

Field experiments and floral trait measurements

For both *Nerine humilis* and *Tritoniopsis revoluta*, single visitations were conducted to assess selection (fitness surfaces) by long and short proboscis insects on mechanical fit traits. One problem with selection studies is that many fail to detect selection in populations with little trait variability (presumably where selection has eliminated less fit phenotypes). To fully explore the shape of the fitness surface, additional variation was introduced to the populations under study by supplementing populations with individuals from other populations. Imported plants were targeted so that we were able to span most of the floral variation found across the entire range of the species (see Schluter 1988).

In single visitation experiments, we used cut inflorescences with at least two flowers in bud, and at the same stage of development. Before offering flowers to pollinators, we

emasculated all experimental flowers in bud and waited between 2 and 3 days for the stigmas to become receptive. Once stigmas were receptive, cut inflorescences containing virgin flowers were placed into test tubes filled with water and mounted on skewer sticks. At each locality, single visitation experiments were conducted between 09:00 and 12:00 when pollinators were most active. Several observers at each of the four localities watched a maximum of 7 emasculated virgin flowers at a time. Observers allowed a pollinator to visit a single flower at a time, after which the visited flower was placed in the shade and gently covered with netting to prevent further visitation by pollinators. Floral visitors were identified by comparing them to a library of known pollinators which were captured after single visits. After each day in the field, the number of conspecific pollen grains on each stigma was counted under a dissection microscope. For *N. humilis* which has exceptionally large pollen grains, we were able to make counts without using stains. In addition, the unmanipulated flowers allowed us to determine female fitness following single visitations for *N. humilis* (see Newman *et al* 2015). For *T. revoluta* we embedded the stigmatic surface in fuschin gel before counting pollen grains.

The following traits associated with the mechanical fit between flower and pollinator morphology were recorded on flowers that received single visitations: Style lengths in *N. humilis* were measured in female phase from the top of the ovary in the nectar chamber to the tip of the stigma and for *T. revoluta*, tube length was measured from the top of the ovary to where the corolla flares. All traits were measured to the nearest millimeter using a set of digital calipers. Pollinator traits were also measured. For insects visiting *T. revoluta*, we measured proboscis length as the mechanical fit trait. Here, we extended the proboscis lengths of captured insects and measured proboscis length as the distance from the base of the proboscis to the tip. However, for *N. humilis*, we measured the functional body length of insects by the angle that insects held their

proboscis whilst foraging (*see* S3.1 – Measurements of functional floral and visitor traits in Newman *et al* 2015), because *N. humilis* does not have a corolla tube which requires a match between insect proboscis length and floral tube length for successful pollination.

Statistical analysis

We explored the role of selection exerted by functionally similar long-proboscid flies or short proboscid insects as well as their combined effects on traits that enhance the mechanical fit between flower and pollinator morphology, by estimating the function related to fitness (pollen deposition) as a function of traits involved in the mechanical fit between flowers and pollinator morphology at each population. To account for the relative abundances of different pollinator species in each analysis, we weighted the number of pollen grains deposited by each species, by their relative abundances recorded in single visitations, thus assuming that all visitors visit long and short tubed flowers with equal probability. To model fitness surfaces of *N. humilis* and *T. revoluta*; different family error distributions were used. This was conducted because we detected zero-inflated measures of pollen deposition and seed set for *Nerine humilis* but not for *T. revoluta*. For *Nerine humilis*, we used generalized additive models (GAM's) with a zero inflated Poisson "ziP" error distribution with an identity link function. For *Tritoniopsis revoluta*, we used cubic spline GAM's with Poisson error distributions with log link functions on splines of individual functional pollinator types. Lambda, which controls the "wiggleness" of the curve, was penalized using REML in these analysis (Wood, 2006). We checked all models for over-smoothing using the function "gam.check" and increased the number of knots (k) in all splines that were over-smoothed. We investigated whether long and/or short proboscid pollinator measurements matched the regions of optimal fitness from additive splines by first determining the range of values for mechanical fit traits, where splines from functionally different pollinator

types overlapped. We then determined the predicted output from long and short proboscid insect GAM models using the “predict.gam” function across 10mm increments between minimum and maximum overlapping measurements for each trait. The overlapping predicted values for each trait at each locality were then summed and overlapping floral traits values were plotted against the summed predicted values to show the full shape of the additive spline. Individual data points from the combined values was then linked using the curvature tool in Adobe Illustrator CC (2017) to illustrate the combined fitness surface from different pollinator groups. All of the above GAM’s were implemented in the R package MGCV (Wood, 2006).

We further obtained standardized directional (β) and quadratic (γ_{ii}) selection gradients from all cubic spline models applied to *N. humilis* and *T. revoluta* using the function “gam.gradients” implemented in the package “gsg” (Morrissey & Sakrejda, 2013). This function averages the first and second-order partial derivatives (directional and quadratic selection gradients) over the slope and curvature of the spline (across the entire distribution of phenotypic values) (*see* Morrissey and Sakrejda (2013) for further details). We were unable to extract selection gradients from splines which we had reconstructed by combining the model predictions from long-proboscid fly and short proboscid insect models, because they do not represent a single linear model from which selection gradients can be estimated in “gam.gradients”. Statistical uncertainty for selection gradients was determined by using case bootstrapping procedures. Doubling of quadratic selection gradients (as emphasized by Stinchcombe *et al* 2008) is already built into the function gam.gradients in the package “gsg” making them comparable to selection gradients calculated by Lande and Arnold methods (Morrissey &

Sakrejda, 2013). All the above statistical analysis were implemented in R version 3.2.3 (Team, 2015).

RESULTS

A total of 388 single visitations were recorded across 2 populations each of *N. humilis* and *T. revoluta*. For *N. humilis*, 147 single visitations were recorded at the short style locality Swellendam where 145 visits were made by honey bees, *Apis mellifera*, and 2 visits made by carpenter bees, *Xylocopa rufitarsus*. At the long style locality; Nuwekloof pass, 72 single visits were made by long-proboscid flies, where 45 of these visits were made by *P. ganglebauri* and 26 by functionally similar *P. longipennis*.

For *T. revoluta*, 55 single visitations were conducted at the long tube locality Barrydale, with 33 of these visits made by the short proboscis insect, *Amegilla spilostoma* and 22 visits by the long-proboscid fly, *P. longipennis*. At the short tubed locality Swartberg; 105 single visits were conducted with 48 visits made by short proboscis insects; 14 of which were from bee's *Amegilla sp* and 28 *Lasioglossum sp* and 6 visits by short proboscis flies from the syrphid family. The remaining 57 single visits were made by an undescribed species of long-proboscid fly.

Natural selection on mechanical fit traits in N. humilis and T. revoluta.

Selection imposed by short and long proboscis pollinators in Nerine humilis

Natural selection exerted by short proboscis insects on style length of *N. humilis* at Swellendam, showed strong negative directional selection for shorter styles for both pollen deposition and seed set responses ($\beta=-0.98\pm0.45$, $P=0.0001$; $\beta=-0.81\pm0.36$, $P=0.018$, Table 4.1, Fig 4.2A, C). We found no evidence for disruptive or stabilizing selection for either pollen deposition or seed set responses ($\gamma=1.27\pm1.46$, $P=0.138$; $\gamma=0.60\pm0.88$, $P=0.46$, Table 4.1, Fig 4.2A, C). At the long style locality (Nuwekloof pass) both directional and quadratic selection gradients were non-significant for pollen deposition ($\beta=0.13\pm0.24$, $P=0.57$, $\gamma=0.17\pm0.59$, $P=0.93$; Table 1, Fig 4.2B). There was no evidence for stabilizing selection on style length when seed set after a single visit was used as a response variable ($\gamma=-0.26\pm1.08$, $P=0.528$, Table 1, Fig 4.2D), however there appeared to be a borderline positive directional relationship between style length and seed set ($\beta=0.64\pm0.33$, $P=0.064$, Table 4.1, Fig 4.2D).

At Swellendam, the morphology of short proboscis honeybees and style lengths of flowers used in single visitations was mismatched for both pollen deposition and resulting seed set splines. This is because as honeybees had shorter functional body lengths than the style lengths of plants used in single visitations (Fig 4.2. A, C). At Nuwekloof pass, the morphology of long-proboscis flies matched style length closely and were in the range of optimal fitness as indicated by the stabilizing selection curve, which was however, non-significant for both pollen deposition and seed set (Fig 4.2. B, D).

Tritoniopsis revoluta

Selection imposed by short proboscis pollinators

Short proboscid pollinators did not exert significant positive directional selection ($\beta=0.13\pm 0.11$, $P=0.29$, Fig 4.3 A, Table 4.2) nor significant stabilizing selection ($\gamma = -0.30\pm 0.08$, $P=0.20$, Fig 4.3A, Table 4.2) on tube length at Swartberg. Similarly, at Barrydale, no selection was exerted by short proboscid pollinators on tube length ($\beta=0.45\pm 0.51$, $P=0.30$; $\gamma = 0.67\pm 2.29$, $P=0.57$, Fig 4.3B, Table 4.2). Since no selection was imposed by short proboscid insects on tube length (Fig. 4.3A & B), there was no association between the morphology of insects and flowers. Insect morphology did not match the regions of optimal fitness at both localities which implies that short proboscid insects exerted no selection on tube length.

Selection imposed by long-proboscid pollinators on Tritoniopsis revoluta.

On their own, long-proboscid flies imposed strong positive directional selection on tube length at both Swartberg and Barrydale ($\beta= 0.43\pm 0.03$, $P=0.0001$; $\beta=1.1\pm 0.087$, $P=0.001$; Fig 4.3 C & D, Table 4.2). There was no signature of stabilizing selection on tube length at Swartberg ($\gamma = -0.004\pm 0.03$, $P=0.98$, Fig 4.3 C, Table 4.2). However, there is weak evidence to suggest that long-proboscid flies may impose disruptive selection on tube lengths at Barrydale ($\gamma = -0.52\pm 0.32$, $P=0.06$, Fig 4.3 D, Table 4.2). At both Swartberg and Barrydale, proboscis length closely matched tube length which matched a fitness optimum at both localities (Fig 4.3 C & D).

Selection imposed by short and long-proboscid pollinators on Tritoniopsis revoluta

Our additive models which summed pollen deposition values across both short and long-proboscid pollinators (Fig.4.3 E & F) were strongly influenced by selection imposed by long-proboscid pollinators at both localities. This result was supported by the proboscis lengths of

long-proboscid flies matching the fitness optima at both localities. In addition, short proboscid pollinators mismatched fitness optima, which is strongly indicative of long-proboscid pollinators driving the overall pattern of selection in both populations.

DISCUSSION

Our results demonstrate that pollinators with different morphology exert divergent selection on floral traits and pollinators with similar morphology exert similar patterns of selection on floral traits. Short and long style populations of *Nerine humilis* with functionally different pollinators displayed contrasting patterns of selection. Similarly, in populations of *Tritoniopsis revoluta* with more than one functionally different pollinator the selective effects of the different pollinators on floral morphology were highly unequal and some pollinators exerted much stronger effects on floral evolution than others. In addition, when the fitness functions of multiple pollinators within *T. revoluta* populations were added together as suggested by Aigner (2001), it appears that a single functional pollinator type (long-proboscid flies) matches the slopes and fitness optima at both Swartberg and Barrydale. Below, we discuss these results in more detail.

Pollinator selection within populations

The two fitness surfaces generated by bees and flies in different *N. humilis* populations were highly divergent (Fig 4.1.). Bees selected for short styles which make better contact with their smaller bodies, despite a mismatch between functional body length of bees and style lengths at Swellendam. In contrast, flies appear to select for longer styles. While positive directional selection by flies on style length was only marginally significant, the trend does make

intuitive sense based on the much longer anatomy of the fly. Furthermore, a previous study demonstrated that populations pollinated by bees versus flies were locally adapted to the pollinators through style length (Newman *et al.*, 2015).

Similarly, for *T. revoluta*, the striking differences in pollinator morphology of bees and long-proboscid flies appeared to generate strong differences in the selection surfaces imposed by each pollinator species. For *T. revoluta*, *Amegilla* bees in both populations typically transferred little pollen per visit and the amount of pollen did not differ much in response to floral trait variation, resulting in relatively flat selection surfaces for floral tube length (Fig.4.3). The reason for this is probably because *Amegilla* bee proboscis length was always shorter than the tube lengths of *T. revoluta*. Consequently, *Amegilla* bees pick up pollen equally (across all floral tube lengths) as they force their way past the reproductive parts of *T. revoluta* in an attempt to extract as much nectar as possible from the long tubes. Furthermore, bee morphological measurements also did not correspond to fitness peaks or inclines on the selection surfaces. These lines of evidence all suggest that although bees play a role in the pollination of *T. revoluta*, they have not had a strong influence on tube length evolution.

This is in contrast to long-proboscid flies visiting the same populations imposing strong directional selection on floral tube length. It was clear that pollen deposition was maximized in flowers with floral tubes that exceeded the proboscis lengths of the visiting flies (as predicted by Darwin 1859, also *see* Muchhala 2009). This is likely to be the result of flies forcing their way past the anthers and stigmas to get the nectar from the bottoms of very long tubed flowers, but theiving the short tubed flowers of nectar without touching the reproductive parts of the flower (Fig 4.3). The two *T. revoluta* populations in this study differed in terms of the species of visiting long-proboscid fly. One species had a very long proboscis while the other had a much

shorter proboscis. Despite their general morphological similarities, it was evident that the selection surfaces differed between species. The fly with the shorter proboscis (23.36mm), produced a selection surface which started to incline sharply when floral tubes were in excess of 15mm, reaching a pronounced peak at 23 mm which corresponded closely with both fly proboscis length and floral tube length in the Swartberg population. Longer tubes did not dramatically increase pollen deposition, perhaps because once floral tube length is longer than fly proboscis length, all flies are forced to make contact with the reproductive parts of the flower when they attempt to obtain the nectar at the bottom of the tube. The selection gradient imposed by this fly was only half the selection gradient imposed by the longer tongued flies from Barrydale (Table 4.2). The selection surface produced by the long-proboscid flies from Barrydale only started to incline sharply when floral tubes were in excess of 55mm. There was no pronounced fitness peak in the Barrydale population, possibly because we were not able to obtain many flowers with tubes which were far in excess of the of the population mean, as we were able to do in the Swartberg (See Fig 4.2 E-F). These results suggest that floral morphology may evolve to enhance the mechanical fit between plant morphology and the morphology of specific pollinators (*sensu* Newman *et al* (2015)). However, they also demonstrate that populations may evolve apparently specialized morphology without reducing the diversity of insect pollinators.

Alternatively, low pollen transfer to short tubed phenotypes may not be a consequence of poor mechanical fit with long-proboscid pollinators but rather due to a mismatch between the positions of pollen placement by the anthers of the native flowers and receipt by the styles of introduced flowers. Here, good stigma contact with the pollinator proboscis would lead to low fitness of short tubed immigrants because pollen from the native population is deposited on the

thorax of the pollinators and not the proboscis. While this is likely to play some role in the low fitness of introduced, short tubed plants. Maladaptation to pollinator morphology is still likely to be the most important reason because *T. revoluta* populations all place pollen on the thorax of long-proboscid fly pollinators (Fig. 4.1). Unpublished data (Minnaar *et al.*, unpublished) also suggests that pollen transfer via the proboscis of long-proboscid flies is much less efficient than pollen transfer via the head or body of long-proboscid flies. Irrespective, our study still demonstrates that functionally different pollinators generate very different fitness surfaces.

Geographic mosaics of pollinator selection

N. humilis clearly experiences divergent pollinator selection environments in the two populations with different pollinator species and it is easy to see how these functionally different pollinators have driven allopatric divergence in style length (Fig. 4.2.). Typically, previous studies have taken examples like this as support for pollinator ecotypes evolving allopatrically in response to the most effective pollinators in each population (Robertson & Wyatt, 1990; Anderson *et al.*, 2010a; Van der Niet *et al.*, 2014b).

However, it is clear that in most cases, plant populations are not visited by a single pollinator and pollinator-mediated selection is more complex than simply determining the effects of the most common or effective pollinators in each population (Aigner, 2001; Aigner, 2004; Sahli & Conner, 2011). Aigner (2001) recognized that plants are likely to respond to the additive effects of different pollinators in a population. By adding the pollen transfer contributions of bees to that of the long-proboscid flies in *T. revoluta* populations, we show that the bees in this system have very little effect on the general shapes of the emergent curves, suggesting that flies

have a much more important role to play in selecting on floral tube traits (Fig. 4.3 E and F). Even if bees transferred much larger pollen loads per visit or if they were more common (i.e. if they were very effective pollinators), it is evident that they would still have almost no effect on tube length evolution in *T. revoluta* because pollen loads do not vary substantially with tube length. The combined fitness surfaces also suggest that differences in the tongue length morphology between the long-proboscid flies at Swartberg and Barrydale are the primary drivers of tube length differences between these two populations. This demonstrates that even in more complex pollinator communities with multiple visitor types, specific types may still have very strong effects on floral evolution and if their abundance differs between communities, then the floral traits of different plant populations are likely to diverge.

Our data is important in visualizing how allopatric divergence may occur between populations which have single or multiple pollinators. It is clear that opposing selection by functionally different pollinators within a population does not necessarily mean that the net selection from multiple pollinator types within a population will be weak (Sahli & Conner, 2011). From these results, we predict that adaptations to specific pollinators occur because those pollinators affect pollen transfer in relation to other floral traits. For example, when pollinated by long-proboscid flies, pollen transfer is enhanced when floral tubes are longer than the pollinator proboscis. On the other hand, it does not matter how effective or efficient bees are at transferring pollen: if pollen is transferred equally to flowers of all tube lengths, bees are unlikely to have strong effects on tube length evolution. To properly understand how floral traits evolve in response to pollinators, it is necessary to isolate the evolutionary contributions of all pollinators to aspects of male and female fitness, instead of ignoring the contributions of so called “less effective” pollinators. This manuscript makes a significant step in terms of isolating

the effects of multiple pollinators in a population, although we note that we have only focused on the female component of fitness, we hope that future work will estimate pollinator effects on both male as well as female fitness components. We challenge researchers to think beyond the outdated and simplistic evolutionary models of floral evolution and divergence which were pioneered by Grant and Stebbins, but which still dominate our perceptions of pollinator driven diversification.

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Table 4.1. Standardized directional and quadratic selection gradients indicating selection on style length in *Nerine humilis*. Estimates highlighted in bold are significant when $P =$ or < 0.06 .

Functional pollinator type	fitness proxy	Locality	floral trait	estimate \pm SE	<i>P</i>
<i>short proboscid insects</i>	pollen deposition	Swellendam	$\beta_{\text{style length}}$	-0.98\pm0.45	0.0001
			$\gamma_{\text{style length}}$	1.27 \pm 1.46	0.14
	seed set		$\beta_{\text{style length}}$	-0.81\pm0.36	0.018
			$\gamma_{\text{style length}}$	0.60 \pm 0.88	0.46
<i>long-proboscid flies</i>	pollen deposition	Nuwekloof	$\beta_{\text{style length}}$	0.13 \pm 0.24	0.57
		pass	$\gamma_{\text{style length}}$	0.17 \pm 0.59	0.93
	seed set		$\beta_{\text{style length}}$	0.64\pm0.33	0.06
			$\gamma_{\text{style length}}$	-0.26 \pm 1.08	0.53

Table 4.2. Standardized directional, quadratic selection gradients indicating selection by functionally different long and short proboscid pollinators on tube length in *Tritoniopsis revoluta*. Estimates highlighted in bold are significant when $P =$ or < 0.06 .

Functional Pollinator type	locality	floral trait	estimate \pm SE	<i>P</i>
<i>short proboscid insects</i>	Barrydale	$\beta_{\text{tube length}}$	0.45 \pm 0.51	0.30
		$\gamma_{\text{tube length}}$	0.67 \pm 2.29	0.57
	Swartberg	$\beta_{\text{tube length}}$	0.13 \pm 0.11	0.29
		$\gamma_{\text{tube length}}$	-0.30 \pm 0.08	0.2
<i>long-proboscid flies</i>	Barrydale	$\beta_{\text{tube length}}$	1.1\pm0.087	0.001
		$\gamma_{\text{tube length}}$	0.52\pm0.32	0.06
	Swartberg	$\beta_{\text{tube length}}$	0.43\pm0.03	0.0001
		$\gamma_{\text{tube length}}$	-0.004 \pm 0.03	0.98



Figure. 4.1. Pollination ecotypes of *Nerine humilis* and *Tritoniopsis revoluta*. demonstrating the mechanical fit between flower and pollinator morphology. The anthers of *Nerine humilis* make contact with the abdomen of A) short and B) long-proboscid insects. For *Tritoniopsis revoluta*, pollen transfer or pick up in C) short proboscid insects and D) long-proboscid flies occurs when pollinators forage for nectar, fitting snugly in the flower gape when they extend their proboscis down the floral tube in search of nectar

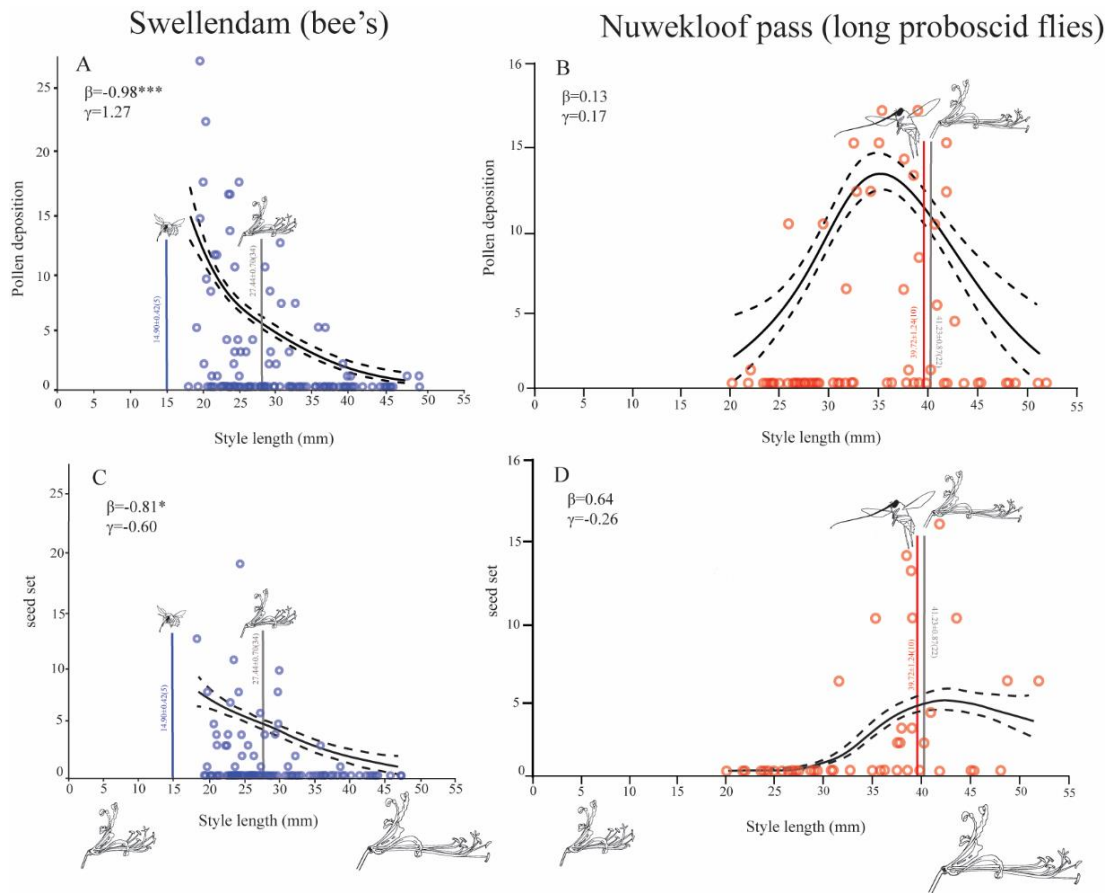


Figure. 4.2. Generalized additive model (GAM)-based visualizations of the form of selection exerted by short proboscis pollinators on style length at Swellendam (A and C) and long-proboscis pollinators at Nuwekloof pass (B and D). Selection gradients extracted from GAM models from Swellendam indicate strong negative directional selection on style length of both pollen deposition and seed set responses (A and C). Which is supported by bee's with shorter mean functional body lengths than mean styles at Swellendam. At Nuwekloof pass, the stabilizing selection signatures was non-significant. However, flower and pollinator morphology matched the fitness optima for both pollen deposition and seed set splines (B and D). Data points represent raw data overlaid onto the GAM visualizations using Adobe illustrator. The solid line indicates the cubic spline and dotted lines represent bootstrap approximations of the standard errors of the GAM predictions.

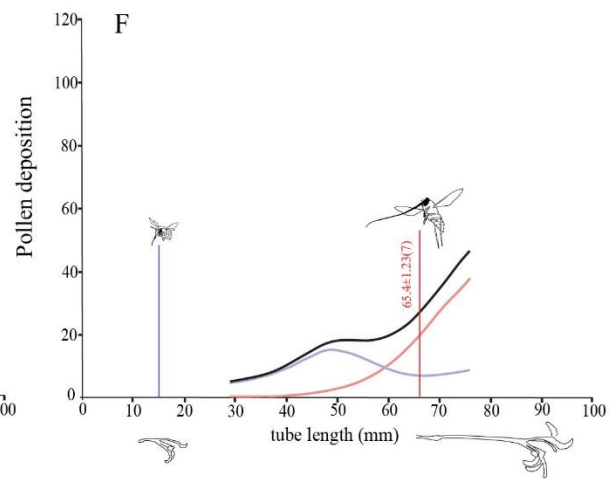
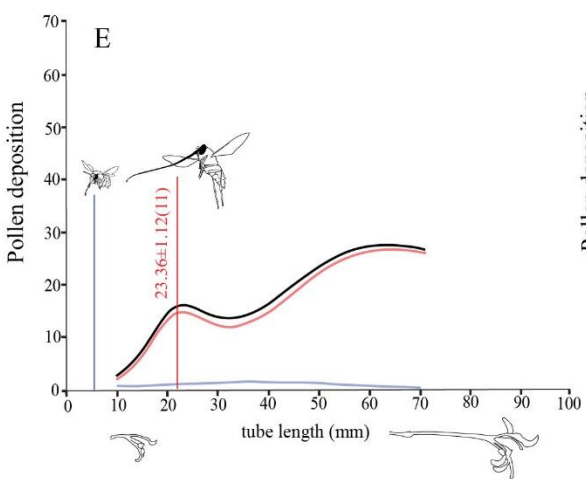
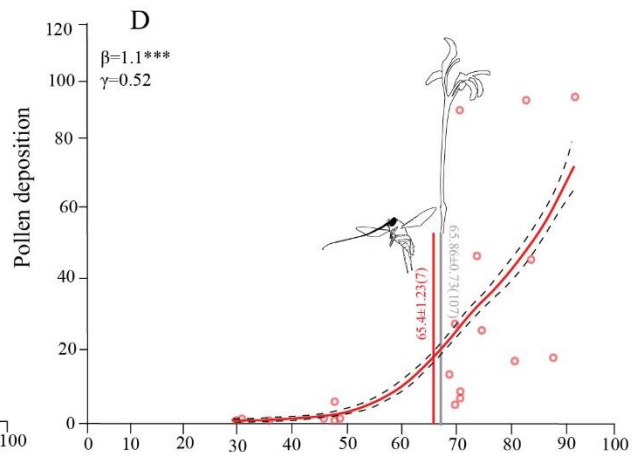
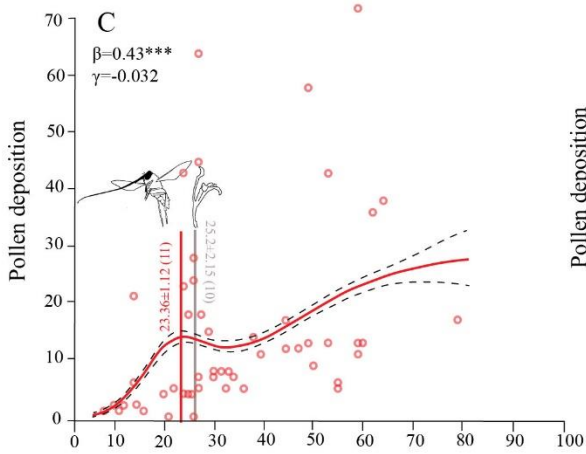
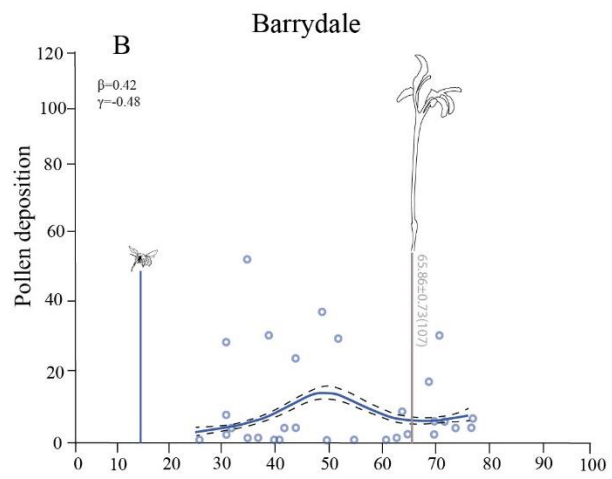
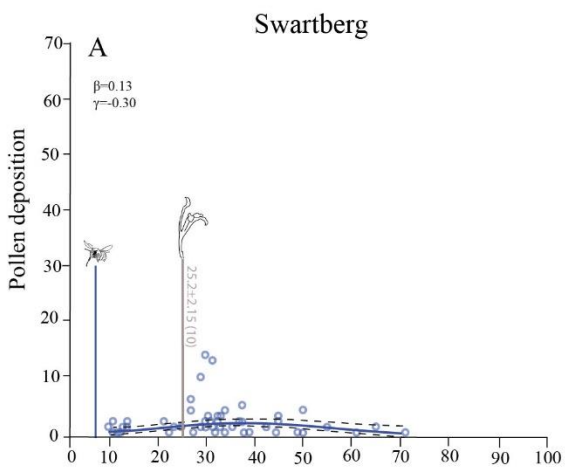


Figure. 4.3. Generalized additive model (GAM)-based visualizations of the form of selection imposed by short and long-proboscid pollinators indicated by blue and red splines on tube length at Swartberg and Barrydale. Selection gradients extracted from GAM models indicate no selection exerted by short proboscid pollinators at either locality, which is supported by the mismatch between flower and pollinator morphology (A & B). However, long-proboscid flies exert significant directional selection on tube length at both localities (C & D) and mean proboscis length's of long-proboscid flies match the fitness peaks and inclines of cubic splines.

The additive effects of short proboscid insects and long-proboscid flies at both localities (E & F), demonstrate long-proboscid flies contributing to overall fitness significantly more than short proboscid insects. In addition, the fitness optima and inclines of the additive splines matches the proboscis lengths of flies rather than bee's at both localities better. Black splines are indicative of the additive effects of short (faded blue spline) and long-proboscid pollinators (faded red spline).



Figure. 4.4. A short proboscis pollinator *Amegilla spilostoma* approaching a long tube flower of *Tritoniopsis revoluta* at Barrydale.

Chapter 5

ECOLOGICAL CHARACTER DISPLACEMENT: STYLE LENGTH VARIATION IN PELARGONIUMS DRIVEN BY GEOGRAPHIC MOSAICS OF PLANT COMMUNITY STRUCTURE

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ABSTRACT

Geographically heterogeneous patterns of selection are thought to be important in generating intraspecific floral variation. Usually geographic mosaics in the pollinator landscape are inferred as the primary agents of divergent selection on floral traits. However, it is also possible that geographic heterogeneity in plant community structure can give rise to divergent selection and consequent intraspecific floral variation. Here we investigate ecological character displacement as the possible driving force behind floral variation in *Pelargonium* communities. First we demonstrate that the co-occurrence of *Pelargonium* species may have negative consequences in terms of interspecific pollen transfer. In particular, we show that the precise area of pollen placement on the body of the pollinator is determined by the amount of style exertion. Using visitation experiments, we demonstrate that individuals from different species experience high interspecific pollen transfer if they have similar style lengths. In contrast, individuals from different species experience less interspecific pollen transfer if they have very different style lengths. Using crossing experiments, we also show that interspecific pollen transfer has negative consequences on seed set, suggesting that co-occurring species should evolve style length differences in sympatry to reduce interspecific pollen transfer. Patterns of style length variation of 8 species of *Pelargonium* over 22 different communities indicate that style lengths are always very different when species co-occur. However, style lengths can be very similar when the same species occur in different populations and do not co-occur. Given the apparently strong selection pressure for competition to drive style length divergence in sympatry, we suggest that character displacement is the most parsimonious explanation for the observed patterns of style length variation in *Pelargoniums*.

INTRODUCTION

Substantial evidence suggests that pollinators are responsible for generating floral variation, when they select upon floral traits involved in attraction, which include scent (Peter & Johnson, 2014; Van der Niet *et al.*, 2014b), flower color (Newman *et al.*, 2012), and traits involved in the efficiency of pollen transfer (Wilson, 1995; Sletvold & Agren, 2010; Newman *et al.*, 2014). When pollinators select on floral traits across a heterogeneous pollinator landscape it can result in local floral forms which have diverged as a result of differences in functional pollinator type (Grant & Grant, 1965; Stebbins, 1970) also known as pollination ecotypes (Robertson & Wyatt, 1990; Van der Niet *et al.*, 2014b; Newman *et al.*, 2015).

However, several authors have cautioned against assuming that floral variation is solely a consequence of selection by pollinators, as floral variation can also arise through non-pollinator agents of selection which include herbivory (Agren *et al.*, 2013; De Jager & Ellis, 2014), pleiotropy, drift as well as phenotypic plasticity (Galen, 1999; Armbruster, 2002; Strauss & Whittall, 2006; Rausher, 2008). An additional, but very seldom explored driver of floral variation is geographic mosaics in plant community structure. One example which does show this is the rewardless orchid *Disa ferruginea* which is locally adapted to the color of the plant communities that it mimics (Newman *et al.*, 2012). *Disa ferruginea* is red when it occurs in communities where its butterfly pollinator drinks nectar from red flowers but orange in communities where its pollinator drinks nectar from orange flowers (Newman *et al.*, 2012).

Interspecific competition is another way in which trait divergence can theoretically evolve and this can lead to trait variation if interacting communities differ geographically. While

competition is frequently thought of as one of the primary drivers of trait divergence, good empirical evidence for it is limited, especially for plants. It is expected that if interspecific competition is important in driving trait divergence, then it should result in a pattern called character displacement (Brown & Wilson, 1956). The hallmark of this pattern is that traits of co-occurring species should be different when they occur in sympatry (because competition drives trait divergence). However those same traits can be very similar when those species occur in allopatric populations and are not competing for resources (Brown & Wilson, 1956; Schluter, 2000). This pattern can also arise through the process of competitive exclusion or ecological sorting (Gause *et al.*, 1934; Connell, 1961) where community structures are determined by pre-adaptation (Schluter & McPhail, 1992; Augustyn *et al.*, 2016). In this scenario, trait variation is not an evolved response to competition. Consequently, to distinguish between the evolution of character displacement and ecological sorting, one needs to determine whether competition could be driving trait divergence in sympatry (Schluter, 2000; Stuart & Losos, 2013).

A recent review by Stuart and Losos (2013) recorded 144 studies testing for character displacement. While 70% of those demonstrated patterns consistent with character displacement, a much smaller fraction (17%) were able to link this pattern to competition. Comparatively speaking, character displacement has only rarely been investigated in plants, and although approximately 20 studies find evidence consistent with character displacement (Beans, 2014), few of these satisfy more than three of the 6 rigorous criteria that Schluter and McPhail (1985) suggested for the demonstration of character displacement.

In plants, character displacement may evolve when different species compete for pollination services from the same pollinator resource. To reduce competition, competing plants may diverge in flowering phenology (Devaux & Lande, 2009). Alternatively, competition for

pollinators can cause divergence in the exploitation of a pollinator's sensory preferences (Hopkins & Rausher, 2012; Grossenbacher & Stanton, 2014; Norton *et al.*, 2015) or the placement sites of pollen on the body of the pollinator (Armbruster *et al.*, 1994; Muchhala & Potts, 2007; Muchhala & Thomson, 2012; Huang & Shi, 2013; Ruchisansakun *et al.*, 2016). In the latter scenario, one would expect heterospecific pollen transfer to be the highest between competing species that have the most similar stamens and stigma's positions (Muchhala & Potts, 2007; Muchhala & Thomson, 2012). Heterospecific pollen transfer can drive divergence in floral traits if it leads to reductions in either male or female fitness. Reductions in male fitness is likely if pollen which could have sired seed is lost to the stigmas of competing species. In contrast, loss of female fitness could occur if heterospecific pollen transfer reduces seed set by obstructing the stigmatic surface (Galen & Gregory, 1989; Proctor *et al.*, 1996), clogging styles with foreign pollen tubes (Scribailo & Barret, 1984; Palmer & Antonovics, 1989) or through the loss of gametes by producing unfit hybrid offspring (Arceo-Gomez & Ashman, 2014; Gomez, 2014).

In the floral communities under study, eight *Pelargonium* (Geraniaceae) species which frequently co-occur are all primarily pollinated by the same long-proboscid fly, *P. longipennis* (Fig. 5.1). *Pelargoniums* visited by these flies have styles and stamens exerted from the top of hypanthia (floral tubes) where the sepals start to flare. Foraging long-proboscid flies insert their proboscides into the hypanthium to access nectar at the bottom of the tube. In doing so, the styles and anthers make contact with the ventral surface of the pollinator's body (*see* Fig. 5.1). The position of pollen placement is determined by how far the stamens are exerted from the mouth of the hypanthium tube (this tends to be slightly longer than pollinator tongue lengths in each population) (Anderson *et al.*, 2010b). Plants with short stamens place their pollen near the head

while plants with long stamens place their pollen near the abdomen. Similarly, the extent of style length exertion is likely to determine the position along the pollinator's body, where pollen is picked up.

Despite the fact that all *Pelargoniums* place pollen ventrally on the pollinator, overlapping pollen placement may be expected to lead to interspecific pollen transfer (IPT). If IPT results in loss of plant fitness, then this is likely to drive the evolution of character displacement. In particular, we expect that species in sympatry will evolve differences in the exertion of styles and stamens to minimize the amount of pollen overlap on the body of the pollinator.

In this manuscript, we will first determine whether there is a mechanism which could possibly drive character displacement in co-occurring *Pelargoniums* (namely loss of fitness due to IPT). Next, we will determine whether patterns of style length variation are consistent with predictions of character displacement. If IPT is driving character displacement, then we predict 1) that similarity in style length is likely to result in pollen overlap on the body of the pollinator and increase levels of IPT for sympatric species. 2) IPT will have negative consequences for one or more interacting species (i.e. IPT will cause reductions in seed set). If both of these predictions are met, there will be grounds to expect a pattern consistent with character displacement. More specifically, we predict that 3) style length of sympatric species will have diverged, whereas style length in allopatric populations may be frequently overlapping. 4) This pattern should occur repeatedly for several populations and species, and should not be attributable to chance or ecological sorting.

METHODS

DOES STYLE LENGTH SIMILARITY LEAD TO INTERSPECIFIC POLLEN TRANSFER?

Two localities (Bosfontein, locality 7 and Suurbraak, locality 8) were chosen as areas of focus because of their abundant pollinator densities and *Pelargonium* species with different pollen colours. At Bosfontein (Figure 5.2A), *P. carneum* has styles of intermediate length [mean±SE (13.77±0.24)] and it co-occurs with short styled *P. pinnatum* [mean±SE (6.02±0.14mm)]. At Suurbraak (Figure 5.2B), *P. carneum* has longer styles [mean±SE (15.45±0.24)] and co-occurs with short styled [mean±SE (5.68±0.17)] *P. pinnatum* plants and intermediate styled [mean±SE (10.82±0.23)] *P. dipetalum* plants. In addition, within each of these populations, different species of *Pelargonium* have different color pollen that can be distinguished under a dissection microscope. At Bosfontein, *P. pinnatum* has light yellow pollen which is also smaller than the brick red pollen of *P. carneum*. At Suurbraak, *P. pinnatum* has yellow pollen, *P. dipetalum* has brick red pollen and 90% of *P. carneum* individuals have yellow pollen and the other 10% light orange pollen, which helped us to distinguish between pollen of *P. carneum* and *P. dipetalum* at Suurbraak.

To determine whether the positions of pollen placement on the bodies of pollinators are associated with style length, we visually compared distributions of style length and pollen placement for each *Pelargonium* species. Seven flies were captured from Bosfontein and four flies were captured from Suurbraak. Flies were killed immediately upon capture using a strong concentration of cyanide. Flies were only killed if we were able to transfer them to the killing jar with a minimum of handling to minimize the displacement of pollen. Immediately after being

killed, afterwards the flies were pinned with their ventral surfaces facing upwards, taking care not to touch any of their ventral surfaces. The body of each fly was divided up into 2mm intervals using a microscope graticule, starting from the base of the proboscis. At each interval, the total numbers of pollen grains were counted under a dissecting microscope and each pollen grain was assigned to a species using colour, size and shape as the diagnostic features.

At Bosfontein and Suurbraak, we performed experiments to determine whether levels of interspecific pollen transfer increased as style length similarity between species increased. At one population (Suurbraak) we increased the natural levels of style length variation for *P. carneum* and *P. dipetalum* by introducing plants from other populations that differed in style length. *P. carneum* plants were introduced from outside populations and had shorter styles than the style lengths at Suurbraak. *P. dipetalum* were introduced from a population with longer styles. In addition, all plants introduced from outside populations had longer hypanthia than native plants. (i.e. if style length/anther length exertion is the unit of selection then we expect that introduced plants should have tube lengths that either match the proboscis of local flies or have tube lengths that are longer than the native plants for certain mechanical fit between the body of the pollinator and style length (Newman *et al.*, 2014)). At Bosfontein, we were unable to introduce plants from outside populations as Bosfontein is one of the earliest flowering populations and no outside populations were in flower at the time of the experiments. One drawback from this experiment is that we could not determine whether low conspecific pollen receipt in introduced plants is the result of the lack of pollen picked up in areas where no pollen is placed by native plants. However, if the pollen of introduced plants were included in the experiment we suspect that introduced individuals would overlap in their placement of pollen with competitors and increase the uptake of interspecific pollen on introduced plants.

All experimental flowers were cut from their field populations after their anthers had dehisced, but before stigmas were receptive. Inflorescences were kept in a cool room until the stigmas became receptive. By the time stigmas were receptive, the anthers had dropped off, ensuring that no self-pollination could occur and also preventing movement of pollen from introduced flowers into natural populations. Once the stigmas on each inflorescence were fully receptive (the stigmas were strongly lobed), we measured style exertion length from the top of where the hypanthium sepals flare to the farthest stigmatic lobe. Following these measurements, we tagged stems of inflorescences with masking tape and wrote the locality of origin and style exertion length of the individual on the tape. On the 6th and 7th of March 2013 at Bosfontein and 27 and 28 March 2014 at Suurbraak, experimental plants were placed in the field early in the morning before pollinators were active in water filled test tubes mounted on skewer sticks. At Bosfontein, 66 individuals of *P. pinnatum* and 33 individuals of *P. carneum* were placed in the field. At Suurbraak, 39 native individuals of *P. pinnatum*, 61 individuals of *P. dipetalum* (40 native and 21 introduced from locality 11) and 51 individuals of *P. carneum* (37 native and 14 introduced from locality 7) were used. Numbers of experimental plants reflected the ratios of co-occurring species in the natural populations. At each locality, *Pelargoniums* occur in immediate sympatry and plants were randomly placed where pollinators could have access to all species in an area of approximately 5 m². All inflorescences were collected at 16:00 and transported back to the field station. On evenings when plants were collected, visited flowers were separated from unvisited flowers (petals of the flowers wilt and fall off after pollen has been transferred after a single visit). We immediately quantified conspecific from heterospecific pollen (pollen from the other *Pelargonium* species) under a dissection microscope after collection, as the stigmatic lobes

start to fold upon one another within a day of being visited and pollen grains from different *Pelargonium* species start to lose their color. Flowers which were not visited were used again the following day. In each study site, no other plant species visited by long-proboscid flies place pollen on the ventral portion of the pollinator's body.

We fitted linear and quadratic slopes to the two populations at Bosfontein where we expect more interspecific pollen transfer of individuals which were most similar in style length to those of competing species, with the proportion of conspecific pollen relative to total pollen grains (conspecific + heterospecific pollen grains) treated as the dependent variable and style-length as the independent variable. At Suurbraak, we also fitted linear and quadratic slopes to each *Pelargonium* species, however *P. dipetalum* and *P. carneum* plants were analyzed with and without the inclusion of the flowers introduced from external populations. Since introduced plants were chosen to increase the styler overlap between species, it is expected that analyses using the introduced plants should more likely have steeper slopes than analyses which do not use introduced plants. In addition, we expect that the species with the shortest styles should have negative slopes (*P. pinnatum*), species in the middle should have a signature associated with stabilizing selection (*P. dipetalum*) and species with the longest styles in each community should have positive slopes (*P. carneum*)

Linear model coefficients were determined using the methods outlined by Lande and Arnold (1983). Quadratic selection coefficients were doubled as emphasized by Stinchcombe *et al* (2008). To overcome the problem of proportion data, we arc-sine root transformed the proportion of conspecific pollen transferred, which was treated as a response variable and style length as independent variable in all of the above analysis. All response and predictor variables

were standardized and relativized for comparative purposes (Lande & Arnold, 1983). All analysis was implemented in R (Core team 2015).

DOES IPT LEAD TO REDUCED SEEDSET?

At a single site (Bosfontein) we performed reciprocal crosses between *P. pinnatum* and *P. carneum*. First, we performed reciprocal interspecific crosses between *P. pinnatum* and *P. carneum* using pollen from at least 3 different individuals per cross. We simulated competition by conducting crosses using mixed pollen loads. Three recently dehisced anthers (each from a different donor) from each of the two co-occurring species were gently rubbed onto the stigma lobes of each pollen recipient. The application of the pollen was alternated so that different species were applied first and last to different donors. For controls, we performed crosses within the same species, again using pollen from at least 3 different individuals for each cross. If IPT has negative consequences on female fitness, we expected that mixed crosses would set fewer seeds than pure intraspecific crosses. Furthermore, if pure interspecific crosses do not form hybrid seed, then this suggests that any morphological patterns associated with divergent styles and anthers across *Pelargonium* communities is the result of ecological character displacement (character displacement without gene flow) and not the result of reproductive character displacement (character displacement with gene flow or reinforcement), which evolves to reduce the incidence of hybrid formation and enhances reproductive isolation upon secondary contact.

Seed-set was counted two weeks after crosses, and fertilized ovules were discerned from aborted ovules which were much smaller and shriveled in appearance. Seed set differences between mixed, heterospecific crosses and conspecific controls were analyzed using Generalized

Linear models with a binomial distribution and a logit link function, which were weighted for overdispersion. To determine statistical differences between heterospecific, conspecific and mixed crosses, we used contrasts with a sequential sidak procedure in SPSS 21 (IBM, Chicago) and values were back transformed from the linear predictor for graphical representation (Fig. 5.3).

IS DIVERGENCE GREATER IN SYMPATRY THAN ALLOPATRY?

During March and April 2013 to 2015, *Pelargonium* community assemblages were sampled by measuring the length of fully receptive styles (*see* methods above) of long tubed *Pelargoniums* at 22 localities (Table S5.1, Fig.5.4.) with *P. longipennis* pollinators (Newman *et al.*, 2014). To visualize morphological patterns associated with character displacement (i.e. species should have different style lengths in sympatry, but they can be similar in allopatry), mean style exertion lengths with 95% confidence intervals were plotted by community on a map of the Western and Eastern Cape of Southern Africa.

CAN SYMPATRIC DIVERGENCE BE EXPLAINED BY CHANCE OR ECOLOGICAL SORTING?

If competitive interactions are responsible for generating character displacement, we expect a pattern of trait overdispersion in the amount of style length exertion displayed by sympatric *Pelargoniums* when mean style length exertions with confidence intervals are plotted as described above. More specifically, style length divergence of sympatric species should be greater than expected by chance. We compared trait divergence in sympatry to randomly assembled null communities using an “evolutionary null model” designed by Muchhala and Potts

(2007). We modified their R script to get rid of duplicated matrices. This model explicitly excludes ecological sorting as a driver of trait divergence. If we find that floral variation of guild communities is explained by local evolution in the null model, then this suggests that our pattern of trait overdispersion cannot be explained by ecological sorting. The study system of Muchhala and Potts (2007) is very similar to our own in that co-occurring species have evolved differences in the lengths of their stamens and styles due to competition for pollen placement site on the bodies of bat pollinators to reduce the negative effects of interspecific pollen transfer.

The evolutionary null model focuses on all widespread species, namely those species that occur in more than one locality, asking what the expected assemblage structure would be if they evolved local exertion lengths at random. The model starts with observed matrix and randomly permutes the non-zero entries for each column of species that occur in more than one locality (columns specify species and rows specify localities). Hence the model preserves the observed structure and does not change the position of the blank cells or identity of species within a single locality. For the above model we generated 1000 null matrices and analyzed their local divergence in the following manner.

First, we calculated the mean difference in the exertion lengths of species pairs adjacent to one another, which was first sorted from the shortest to the longest exertion length within each locality (e.g. in a habitat with 3 species we calculated differences in mean style exertion length between the first and the second, and then between the second and the third). We then calculated the mean style length difference for each matrix (observed and nulls) and generated a frequency distribution of this value for the 1000 null matrices and compared the observed mean differences with this frequency distribution; if our results are statistically significant, then we expect that less than 5% of the nulls will show a higher than average difference (*see* Muchhala and Potts 2007

for additional information). If our results are non-significant it means that species have maintained their exact positions across multiple localities and do not differ in the length of their styles in the context of their communities.

RESULTS

DOES STYLE LENGTH SIMILARITY LEAD TO IPT?

Tube length histograms of co-occurring Pelargoniums from Bosfontein and Suurbraak (Fig. 5.2A and B) all have different peaks within a site. The tails of these histograms overlap at Suurbraak (Fig. 5.2B) which has three sympatric Pelargoniums but not at Bosfontein which only has two (Fig. 5.2A). Style lengths of *P. carneum* are long at the Suurbraak site where they co-occur with *P. dipetalum* which has an intermediate style length. They are shorter at Bosfontein where there are no competing species with intermediate style length. The placement of pollen on fly bodies was matched by the observed style length exertions of the different species, leading to some areas on the pollinators' bodies with pure pollen loads and other areas with overlapping pollen placement. At Bosfontein, *P. pinnatum* pollen was placed on the chin of the fly, showing very little overlap with the placement of *P. carneum* towards the thorax and abdomen of the fly (Fig. 5.2C). However, at Suurbraak, we found a higher degree of overlap in pollen placement between the three species (Fig. 5.2D). In particular, we found that the short styled *P. pinnatum* places pollen on the chin of the fly, overlapping in pollen placement with longer styled *P. dipetalum* which places pollen on the thorax of the fly. Notably, the pollen placement of *P. carneum* differs between the two sites: at Suurbraak, the longer styled *P. carneum* only places pollen on the abdomen of the fly whereas at Bosfontein, *P. carneum* also places pollen on the thorax of the fly (Fig. 5.2D).

At Bosfontein we found a significant negative directional association between style length and the purity of pollen loads for *P. pinnatum* ($\beta=-0.09\pm 0.03\text{SE}$, $P=0.02$, Table 5.1, Fig. 5.2E). As style length increased and styles became more similar to the lengths of co-occurring *P. carneum*, the purity of their pollen loads decreased. Quadratic relationships for *P. pinnatum* were not significant ($\gamma=0.04\pm 0.02\text{SE}$, $P=0.44$, Table 5.1, Fig. 5.2E). *Pelargonium carneum* on the other hand showed no significant directional ($\beta=0.1\pm 0.09\text{SE}$, $P=0.27$, Table 5.1, Fig. 5.2E) or quadratic association with an increase in style length ($\gamma=-0.14\pm 0.02\text{SE}$, $P=0.64$, Table 5.1, Fig. 5.2E).

At Suurbraak, we found no significant associations between style length and purity of the pollen loads for *P. pinnatum* ($\beta=0.13\pm 0.07\text{SE}$, $P=0.07$, $\gamma=-0.06\pm 0.07\text{SE}$, $P=0.66$, Table 5.1, Fig. 5.2F). However, a significant positive directional selection between style length and the purity of pollen loads was detected for *P. dipetalum* at Suurbraak ($\beta=0.27\pm 0.09\text{SE}$, $P=0.005$, Table 5.1, Fig. 5.2F). No significant quadratic associations were detected ($\gamma=0.05\pm 0.13\text{SE}$, $P=0.86$, Table 5.1, Fig. 5.2F). We were unable to detect any significant associations between *P. carneum* pollen purity and style length ($\beta=0.12\pm 0.11\text{SE}$, $P=0.27$, $\gamma=-0.34\pm 0.09\text{SE}$, $P=0.07$, Table 5.1, Fig. 5.2F).

When individuals of *P. dipetalum* were introduced from outside populations to extend the phenotype, we found a significant quadratic association between the purity of pollen and style length where the purity of *P. dipetalum* pollen at the extremes are reduced ($\gamma=-0.24\pm 0.05$, $P=0.025$, Table 5.1, Fig. 5.2F). No directional selection was detected for this species despite the addition of additional phenotypic variation ($\beta=0.03\pm 0.06$, $P=0.63$, Table 5.1, Fig. 5.2F). After individuals of *P. carneum* were introduced, *P. carneum* showed a significant positive association between style length and pollen purity ($\beta=0.3\pm 0.08$, $P=0.001$; $\gamma=-0.16\pm 0.07$, $P=0.22$, Table 5.1,

Fig. 5.2F). Here, more heterospecific pollen is found on the styles at the short end of the extreme where there is overlap with *P. dipetalum* style length.

DOES IPT LEAD TO REDUCED SEED SET?

Mixed cross pollinations resulted in significantly decreased seed set in *P. pinnatum* as well as *P. carneum* when compared to conspecific crosses ($t=2.24$, $DF=35.93$, $P=0.032$; $t = 6.35$, $DF=82.92$, $P<0.0001$, Fig.5.3). Heterospecific crosses between *P. carneum* and *P. pinnatum* resulted in zero seed set between species.

IS THERE EVIDENCE FOR A PATTERN CONSISTENT WITH CHARACTER DISPLACEMENT?

There is significant interspecific and intraspecific style length variation within and among *Pelargonium* communities and intraspecific variation is particularly evident in the widespread species; *Pelargonium pinnatum*, *P. carneum*, *P. dipetalum* and *P. myhrifolium* (Fig. 5.4.). In addition, at all localities containing more than two species, style lengths appear to be spaced so that overlap between co-occurring species is minimized. The pattern observed also suggests that shifts in style length are driven by the presence of co-occurring community members. For example, when *P. dipetalum* occurs without *P. carneum*, their styles are frequently overlapping (compare site 11 for *P. dipetalum* with sites 10, 5, 14, 2, 15 and 17 for *P. carneum*, Fig. 5.4). However, in all three instances where these two species co-occur (sites 8, 9, 10, Fig. 5.4), there is no overlap in style length, and the style length of *P. dipetalum* seems to be reduced in length relative to site 11 (Fig. 5.4). In these three populations, *P. carneum* populations have long styles relative to many other sympatric *P. carneum* populations and it is also possible that they may have lengthened their styles in response to *P. dipetalum*. Similarly, several *P. carneum* and *P.*

myhrifolium populations frequently have similar style lengths when they occur in different populations. Most notably the *P. carneum* populations with the longest style lengths (sites 1, 3, 8, 19, Fig. 5.4) all occur in the absence of *P. myhrifolium*, which usually has a slightly longer style than *P. carneum*. *P. carneum* style lengths in these four populations are very similar to the style lengths of many *P. myhrifolium* populations (sites 14, 15, 16, 20, 21, 22, Fig. 5.4).

However, *P. myhrifolium* was not found in communities in the western cluster of sites which makes it hard to confirm this pattern across the entire system. Nevertheless, there are only six sites where *P. carneum* and *P. myhrifolium* co-occur in the eastern cluster of sites (sites 12-17, Fig. 5.4). Within each of these sites, the style lengths of the two species appear to be very clearly differentiated, with very little overlap. In all 6 of these sites; *P. myhrifolium* appears to have long styles relative to the three *P. myhrifolium* populations which have no *P. carneum* plants (sites 20, 21, 22, Fig.5.4). This suggests that co-occurrence may have driven a shift in *P. myhrifolium* styles. Similarly, *P. carneum* has some of the shortest style lengths recorded for the species in four of the sites of coexistence (sites 12, 13, 14, 16, Fig.5.4), suggesting that it may also have shifted its style length in these sites.

CAN SYMPATRIC DIVERGENCE BE EXPLAINED BY CHANCE OR ECOLOGICAL SORTING?

We found a pattern consistent with trait overdispersion when visualizing the plotted community members. This pattern of trait overdispersion can be attributed to local divergence in style length, as the evolutionary null model was highly significant with only 6 of the thousand generated matrices having mean style length exertion differences higher than the observed mean style exertion length difference of 5.85 ($P=0.006$). The evolutionary null model had a mean exertion length difference of 5.3 ± 0.24 SD (4.69-5.95, min-max).

DISCUSSION

Several lines of evidence demonstrate processes and patterns consistent with ecological character displacement. Specifically, our results suggest that variation in the style lengths of widespread species are driven by competition for pollen placement. We show that style length exertion determines where pollen is placed on the body of pollinators and that individuals of different species experience high interspecific pollen transfer if they have similar style lengths (. In contrast, heterospecific individuals experience low levels of interspecific pollen transfer if their style lengths are more different. Using crossing experiments, we also demonstrate that IPT results in a reduction of female fitness. These processes lead to the hallmark patterns associated with character displacement: Species always have strong style length differences in sympatry but they can have overlapping style lengths when they occur in allopatry. Importantly, this pattern is replicated across multiple species and over multiple populations and an evolutionary null model demonstrates that this pattern is unlikely to have resulted by chance or ecological sorting.

While several studies purportedly demonstrate character displacement by showing the overdispersion of character traits (Dayan *et al.*, 1990), Schluter and McPhail (1985) emphasize that this pattern can have several alternative origins including ecological sorting. They suggest that studies need to satisfy six criteria for the demonstration of character displacement. These criteria are: 1.) Shifts in resource use (pollen placement) should match a shift in phenotype (style length) 2.) Evidence demonstrating competition between similar phenotypes is required. 3.) A pattern of character displacement should not be the result of chance. 4.) Differences between species or populations must represent evolutionary shifts and not just species sorting. 5+6.)

Differences between traits across populations should not be environmentally controlled and the traits under selection by competition should be heritable. Our study is one of a handful of studies (Fishman & Wyatt, 1999; Caruso, 2000; Muchhala & Potts, 2007; Hopkins & Rausher, 2012; Grossenbacher & Stanton, 2014; Norton *et al.*, 2015) that demonstrate more than 3 of these criteria for angiosperms. Below, we contextualize our study in terms of each of these 6 criteria:

1) *A shift in resource use should match a shift in phenotype.*

We clearly show a link between resource use and style length at Bosfontein and Suurbrak. Here, we show that style length exertion determines where pollen is placed on the pollinator. We also show that when the style lengths of *P. carneum* differ between these two sites it results in differences in the placement of pollen on the body of the pollinator (Fig. 5.2. C & D). Few studies have investigated differential pollen placement in plant communities sharing the same pollinator (Muchhala & Thomson, 2012; Huang & Shi, 2013) and we are not aware of any other studies that show differential pollen placement across multiple communities in association with shifts in style length.

2.) *Evidence demonstrating competition between similar phenotypes*

If character displacement through pollen placement drives shifts in floral morphology, then one should expect that style length is linked to plant fitness. Our study establishes this link by showing that heterospecific individuals experience more interspecific pollen transfer if their styles are similar in length than if they are different in length. Furthermore, because we demonstrate a fitness cost associated with the purity of pollen loads, we argue that this metric can be used as a surrogate for female fitness for our field experiments. Selection studies are frequently unable to pick up patterns of selection in natural populations, because when selection

is strong, it would have already eliminated most phenotypes of low fitness (Schluter, 1988). By extending the style length phenotype and introducing individuals from outside populations we were able to circumnavigate this problem. Consequently, analyses using introduced individuals allowed us to demonstrate selection against phenotypic similarity more easily. Overall, this suggests that different species compete for pollen placement sites on a finite resource (the body of the pollinator) with increased intensity of competition when heterospecific individuals have similar style lengths. In addition, we also show that mixed pollinations and interspecific crosses result in no hybrid seed which is consistent with the process of ecological character displacement where species in sympatry have completed reproductive isolation. In contrast, if mixed and interspecific crosses led to hybrid seed set formation then divergence of floral traits within communities would reflect reproductive isolation upon secondary contact (Scott Armbruster & Muchhala, 2009), a process known as reproductive character displacement or reinforcement (Hopkins, 2013).

3.) and 4.) A pattern of character displacement should not be a result of chance and should be the result of evolutionary shifts.

Across the 22 *Pelargonium* communities, we found a strong pattern consistent with character displacement (Fig. 5.4): Species that are widespread appear to show shifts in style length in association with community context. Importantly these shifts occur across multiple species, and over multiple sites and a null model suggests that these patterns are unlikely to have resulted by chance. The null model was in fact highly constrained so that ecological origins of the pattern were excluded, suggesting an evolutionary origin of the pattern. Armbruster *et al* (1994) also used a similar null model to exclude the possibility that ecological sorting was the driving force behind patterns of trait overdispersion in Australian trigger plants (*Stylidium*). In addition to the

null model which suggests that style length variation has an evolutionary origin, we also show that style length appears to be under selection mediated by competition. Here style length phenotypes which differ most from co-occurring species receive less interspecific pollen transfer than phenotypes which are similar to co-occurring species and this results in reductions in female fitness.

5.) and 6.) *Differences between traits across populations should not be environmentally controlled and traits under selection by competition should be heritable.*

Environmental control and heritability were two criteria which were not explicitly tested in this study. While environmental variables may potentially generate trait variation, we find it unlikely to result in a pattern consistent with character displacement, because variations in the abiotic environment are more likely to affect morphological traits of closely related species in a similar way. That is, instead of diverging in sympatry as they do in our study, different species within a site are more likely to exhibit style length shifts in the same direction. Furthermore, populations which show style length shifts are often in very close proximity (< five km), and they often occur on the same soil types and experience similar rainfall. The main differences between these sites are less likely to be related to soil or moisture, but rather the presence or absence of certain community members. For example, *P. carneum* in communities 8 and 2 (Fig.5.4) have different style lengths but yet the populations occur less than five km apart, they share similar climatic conditions (EL unpublished), and they grow on the same soil (Ruens shale Fynbos). The two communities however, differ in the absence and presence of *P. dipetalum*. Heritability is another necessity if traits like style length are to respond to selection. While we did not test whether style length in *Pelargonium* is heritable, all traits under selection display heritability (Harder & Johnson, 2009). Several studies demonstrate that style length is heritable

in other systems (Carr & Fenster, 1994; Andersson, 1996; Motten & Stone, 2000) and style length is often linked to floral tube length, which has also frequently been shown to be heritable (Anderson *et al.*, 2016b). We conclude that it is very unlikely that style length does not have a heritable component.

In our study we found that co-occurring plants compete for pollen placement sites on the body of a shared pollinator, which represents a resource. The competitive exclusion principle suggests that competition for the same resource will lead to the competitive exclusion of all but one competing species unless the resource is divided into more fine-scale niches (niche differentiation) (Gause *et al.*, 1934; Connell, 1961). In our case, plants appear to divide the body of the pollinator into different niches, so that within a site, different species specialize on placing pollen on different parts of the pollinator and in so doing, they alleviate competition. Similarly *Anolis* lizards specialize and utilize different parts of trees to reduce competition (Losos, 2009). Notably different populations of the same *Pelargonium* species place pollen on different parts of the pollinator depending on which other community members they are competing with. We suggest that the most plausible explanation for the variation of style length between populations is character displacement.

Table 5.1. Linear and quadratic model coefficients of univariate pollen purity analysis, from sympatric *Pelargonium* species at Bosfontein and Suurbraak. Bolded values indicate significance.

locality	species	fitness function	coefficients	t	estimate±SE	<i>P</i>	AIC
Bosfontein	<i>P. pinnatum</i>	natural	β	-2.44	-0.09±0.03	0.02	35.12
			γ	0.76	0.04±0.02	0.44	36.52
	<i>P. carneum</i>	natural	β	1.13	0.1±0.09	0.27	53.51
			γ	-0.48	0.14±0.02	0.64	55.27
Suurbraak	<i>P. pinnatum</i>	natural	β	1.88	0.13±0.07	0.069	38.34
			γ	-0.44	-0.06±0.07	0.66	40.13
	<i>P. dipetalum</i>	natural	β	2.97	0.27±0.09	0.005	55.12
			γ	0.18	0.05±0.13	0.86	57
	<i>P. dipetalum</i>	ancestral	β	0.49	0.03±0.06	0.63	95.14
			γ	-2.3	-0.24±0.05	0.025	91.82
	<i>P. carneum</i>	natural	β	1.11	0.12±0.11	0.27	51
			γ	-1.88	-0.34±0.09	0.07	49.36
<i>P. carneum</i>	ancestral	β	3.49	0.3±0.08	0.001	99.29	
		γ	-1.25	-0.16±0.07	0.22	99.64	

Table. S5.1. Mean style length exertions with standard errors and population sizes of 22 Pelargonium communities across the Western and Eastern Cape of Southern Africa.

Locality	<i>P. carneum</i>	<i>P. myhrifolium</i>	<i>P. zonale</i>	<i>P. laevigatum</i>	<i>P. pinnatum</i>	<i>P. dipetalum</i>	<i>P. luteolum</i>	<i>P. tetragonum</i>
1	16.73±0.35(28)							
2	13.77±0.24(33)							
3	16.37±0.35(9)							
4	14.37±0.28(20)				7.15±0.3(19)			
5	14.34±0.63(9)				8.16±0.37(5)			
6	12.88±0.64(4)				7.25±0.32(2)			
7	13.77±0.23(94)				6.02±0.14(65)			
8	15.45±0.24(34)				5.68±0.20(53)	10.82±0.27(44)		
9	15.13±0.18(61)					10.61±0.62(4)		
10	14.2(1)					8.66±0.24(12)		
11						13.98±0.20(28)		
12	12.02±0.92(5)	17.91±0.34(19)						
13	13.06±0.2(24)	18.18±0.33(19)						
14	13.18±0.36(18)	17.35±0.48(7)						
15	14.44±0.47(13)	17.58±0.22(17)						
16	13.87±0.37(4)	16.68±0.86(9)						
17	14.62±0.63(11)	17.91±0.48(25)					5.96±0.17(17)	
18	14.56±0.33(24)						5.02±0.18(18)	
19	16.12±0.44(19)			5.93±0.18(17)				
20		16.16±0.2(19)	10.57±0.24(19)	5.32±0.28(14)				28.91±0.88(23)
21	16.77±0.25(24)							
22		17.97±0.56(22)						



Figure 5.1. The long proboscis fly *Prosoeca longipennis* visiting receptive flowers of *Pelargonium carneum* at Bosfontein (photo: Bruce Anderson).

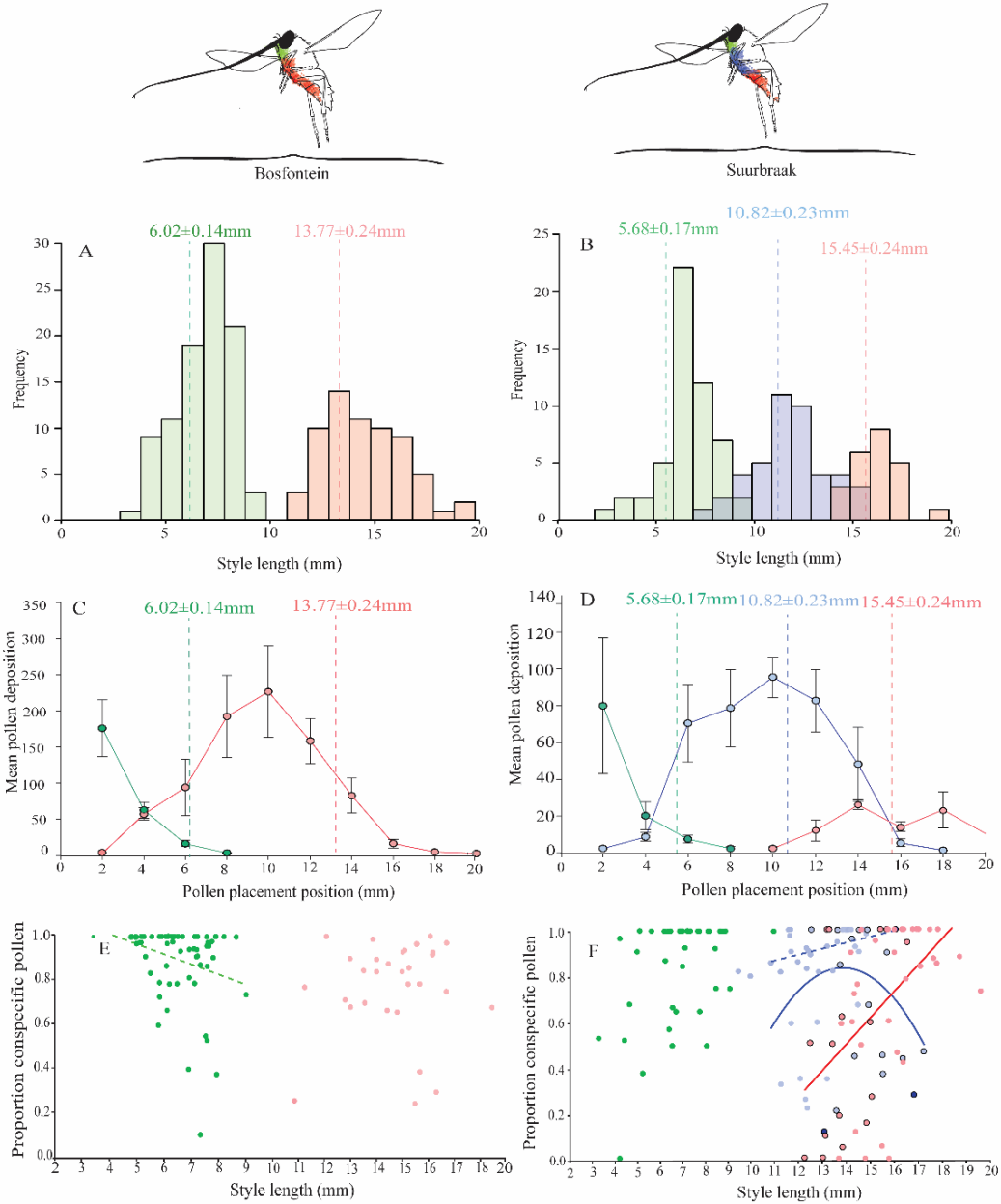


Figure 5.2. In the above figure, different colours denote different *Pelargonium* species. Namely *Pelargonium pinnatum* (green), *P. carneum*, (red) and *P. dipetalum* (blue). A and B illustrate differences in style length variation among communities, demonstrating very little to no overlap in style length at Bosfontein with two species, and increased overlap in style length at Suurbraak with three species. This variation in style length is matched by the mean placement of pollen with standard errors on the bodies of long-proboscid flies' which was measured in intervals of 2mm each (C and D). Pollen placement is then followed by an analysis investigating pollen purity, whereby coloured data points indicate different *Pelargonium* species and black circles around data points refer to introduced individuals from outside populations. Stippled regression lines indicate significant selection on the natural phenotype and solid lines indicate significant selection on the inflated phenotype. These figures suggest that the differential placement of pollen is the driver of floral variation through competition within *Pelargonium* communities (E and F). Illustrations indicating regions of pollen placement on long-proboscid flies are shown at the top of the figure under each locality.

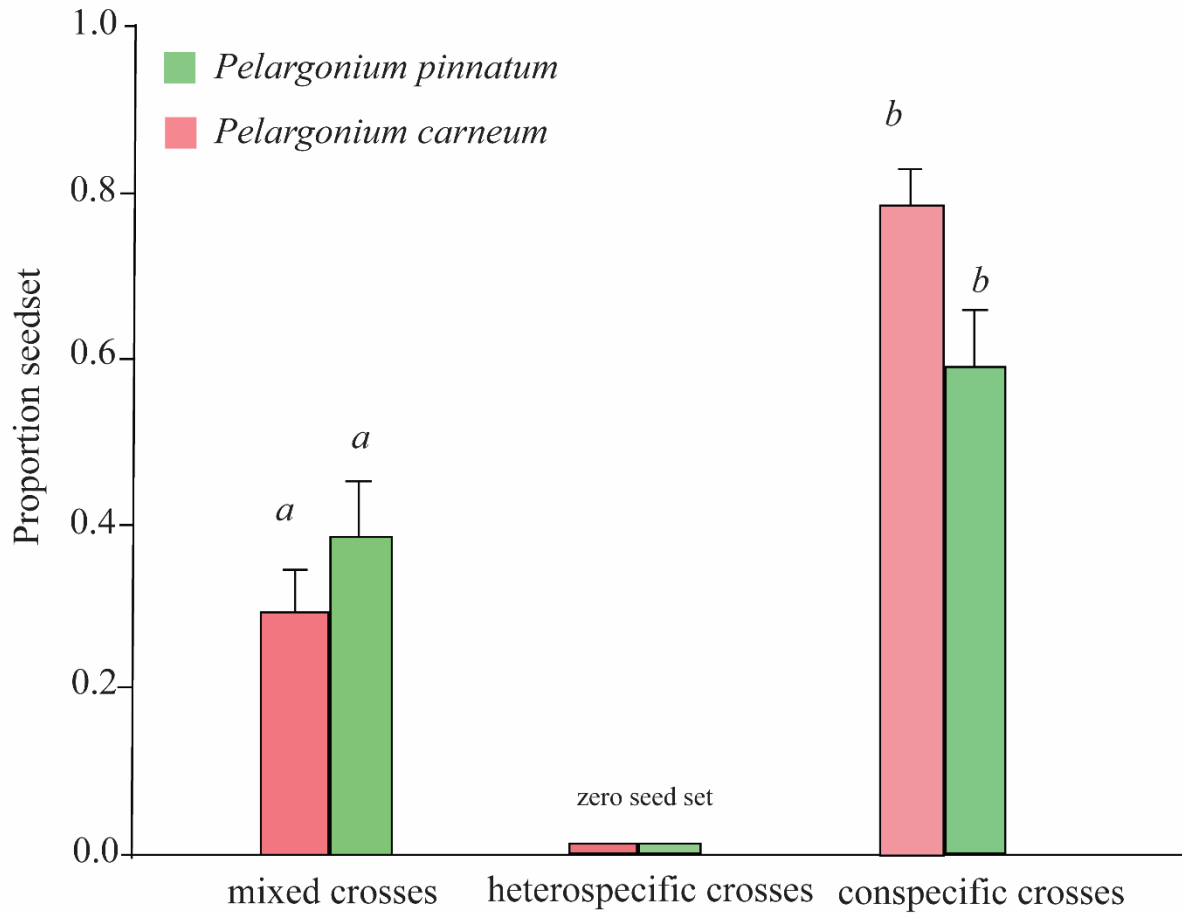


Figure 5.3: Proportion seed sired from mixed crosses, heterospecific crosses and conspecific crosses between and within *Pelargonium pinnatum* and *P. carneum* at Bosfontein. Letters *a*, *b* and *c* indicate significant between treatments. Notice that heterospecific crosses did produce hybrid seed.

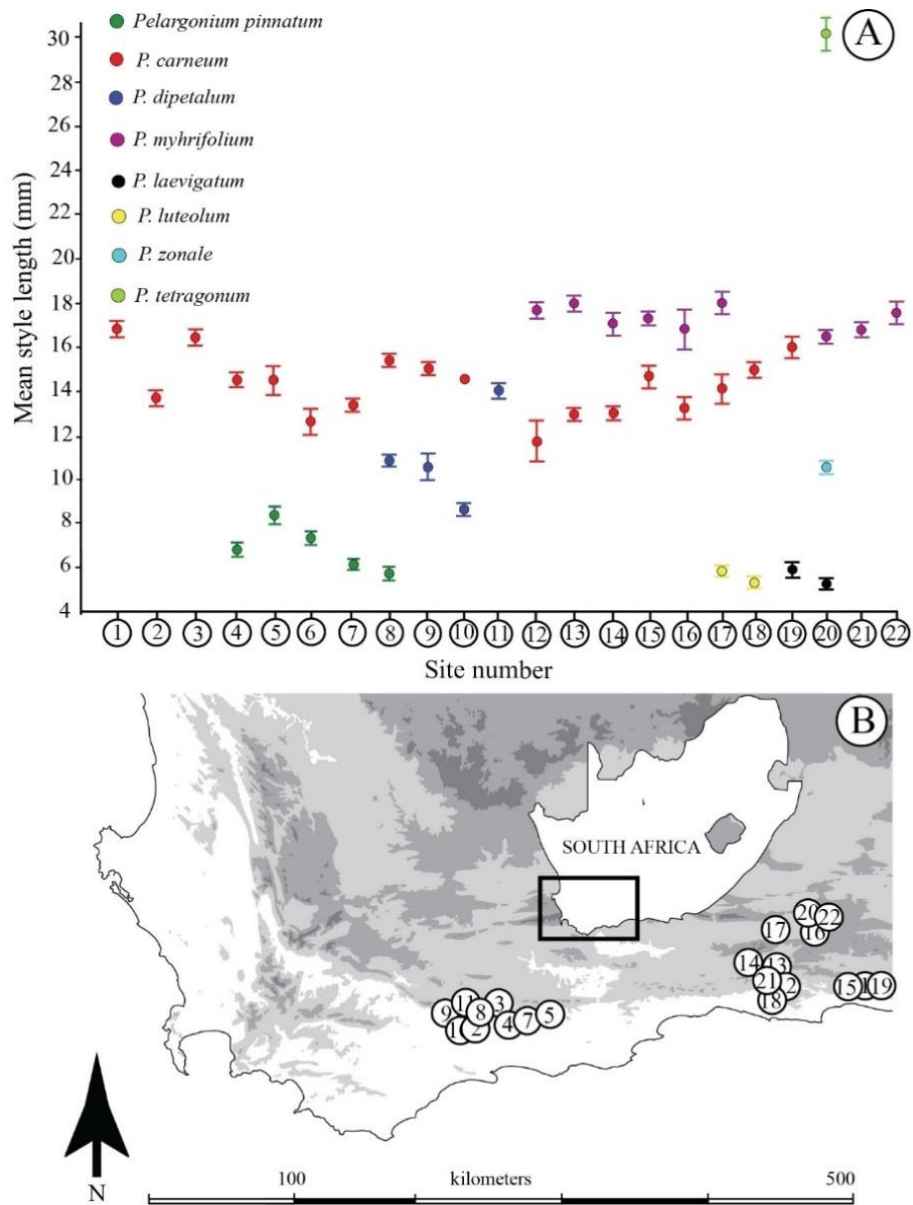


Figure 5.4. The above figure illustrates mean style length with 95% confidence intervals plotted for 22 *Pelargonium* communities in the Western Cape and Eastern Cape of South Africa. *Pelargonium* communities in A are linked to their geographic locations indicated on the map in B.

Chapter 6

CONCLUSION

The results of this thesis suggest that the heterogeneity of both the pollinator landscape and the structure of plant communities can play an important role in driving floral divergence. This is evidenced by what appears to be adaptive variation in floral form in response to variation in pollinator and plant communities, and I have referred to these variants as ecotypes (Armbruster, 1985; Robertson & Wyatt, 1990; Anderson *et al.*, 2010a; Boberg *et al.*, 2014; Van der Niet *et al.*, 2014a). Within the ecological speciation continuum, these ecotypes represent the initial stages of the speciation process, before the formation of fully reproductively isolated species and is a clear example of contemporary evolution (Nosil, 2012; Ellis *et al.*, 2014). Studying ecotypes allows researchers to identify the agents of selection which are important in the initial stages of the speciation process, as it is difficult to determine which agents of selection were important if taxa are already well-established (Coyne & Orr, 2004). Within the context of this thesis; below I attempt to dissect the mechanisms behind pollinator and community driven ecological divergence in the *P. longipennis* system and in the final paragraphs, I will contextualize the roles played by heterogeneity in the pollinator and community landscape in the diversification of plant populations.

ECOLOGICAL DIVERGENCE DRIVEN BY POLLINATORS

Within the *P. longipennis* pollination system, adaptation to pollinators may drive ecological divergence through two main processes, unilateral evolution and coevolution. Unilateral

evolution is when one species adapts to a second species, but there is no evolutionary response of the second species to the first. Perhaps the clearest example of this process can be found in chapter three where *N. humilis* is unlikely to have influenced fly proboscis length very much. The flowers of *N. humilis* do not have any corolla tubes and so even short proboscid insects can easily reach the nectar. However, their styles and anthers are exerted in a manner which matches the total length of foraging insects (proboscis plus body) (Newman *et al.*, 2015). Clear evidence of plant adaptation to the pollinators is seen in local adaptation experiments showing that no contact is made between pollinators and plant reproductive organs when floral parts do not match insect body lengths. Consequently, the flowers appear to have adapted to the functional body lengths of the pollinators but the pollinators need not adapt to the floral form to access the nectar. Large shifts in plant ranges beyond the range of the fly, are also likely to expose plants to novel pollinators, and plants may be forced to adapt to those novel pollinators (Newman *et al.*, 2014). Because those novel pollinators are already adapted to floral communities, the novel guild members may exert only very weak selective pressures on those pollinators. Such range shifts may then result in very large changes in floral traits with very little reciprocal change in the traits of the pollinators and I expect that this may have occurred in the many examples illustrated in this thesis of species exhibiting pollination ecotypes (*see* Chapters two, three and four). However, this contrasts to how floral divergence occurs in floral guild communities visited exclusively by *P. longipennis* in that reciprocal selection between proboscis length and flower depth is likely to be the most important process driving floral variation (*see* Chapter two). Coevolution, the second process driving floral adaptation occurs when changes in one species drive changes in a second interacting species and vice versa. Darwin envisaged that long tubed flowers and their long tongued pollinators are likely to coevolve in this manner. Two separate

studies on long-proboscid flies and long tubed flowers appear to have demonstrated that the pollinators and plants are coevolved (Pauw *et al.*, 2009; Paudel *et al.*, 2016). Using single visitation experiments, they showed that both the efficiency of pollen transfer and the amount of nectar extracted by the flies was mediated by the match between proboscis length and floral tube length. Coevolutionary races can lead to trait variation if they proceed to different end-points in different populations (Anderson *et al.*, 2010b; Anderson, 2015). For example, coevolutionary races between pollinator mouthpart lengthening and floral tube lengthening are expected to slow down or stop when the benefits of increased length are outweighed by the costs of increased length. In addition to simple construction costs, these costs may include difficulties for flight or maneuverability in the case of insects or risk of breakage in plants (*see* Anderson and Johnson 2008). Importantly, these costs are likely to vary geographically in response to nutrient availability, wind or temperature and consequently abiotic factors may have an important role to play in generating variation in the endpoints of coevolutionary races (Anderson & Johnson, 2008; Newman *et al.*, 2014; Toju, 2014). While I did not test for reciprocal selection in this thesis, I was able to show that plant traits are frequently locally adapted to the traits of their pollinators, leaving the question of whether the pollinator traits have adapted to the floral traits. The *P. longipennis* populations with the longest mouthparts were all populations where the flies foraged from just a single nectar source: *T. revoluta* populations with equally long tubes. Since these flies have not been observed using any other nectar sources in those populations and the flies use their proboscides for the sole purpose of consuming nectar; it would seem reasonable to assume that the extreme length of their proboscides in those populations is the result of adaptation to *T. revoluta*. While this is highly suggestive of pairwise coevolution, the flies visit multiple plant species in other populations. However, this does not mean that coevolution does

not occur in sites with multiple plant species. As the sole pollinator of several plant species within a population, I expect that each of those long-tubed plant species will be selecting for proboscis length in a similar manner and the sum of those selection surfaces is likely to result in considerable selection for increased proboscis length (Anderson, 2015). In all likelihood, some guild members may influence fly tongue length more than others. In particular, common species, or species with high rewards may be very strong drivers of fly tongue length, while rare or rewardless species may play little or no role in selecting on fly tongue length (Anderson, 2015). Nevertheless, because plant fitness is dependent on trait matching, even rare or rewardless guild members are forced to track, or are dragged along by coevolutionary races between flies and other guild members (Anderson *et al.*, 2005b; Anderson & Johnson, 2009).

ECOLOGICAL DIVERGENCE DRIVEN BY PLANT COMMUNITIES

Coevolution is also one of the most important processes driving character displacement. In plant communities pollinated by long-proboscid flies, competition for placement sites leads to reciprocal selection on style length. Reciprocal selection selects for differences in style length, so that pollen interference is minimized and this results in overdispersion of the trait within communities. As a coevolutionary response, co-occurring community members often appear to have evolved shifts in style length relative to the style lengths of the same species when they do not co-occur. Coevolutionary divergence evolved through character displacement differs from coevolutionary divergence in mechanical fit between tube length and proboscis length, because the former is based on an antagonistic or competitive interaction, whereas the latter is based on a mutualism (Thompson, 2005). That said, it is possible that both processes can act in concert to drive floral divergence in *Pelargonium*. In this scenario, coevolution drives divergence by

mechanical fit on tube length and antagonistic coevolution drives divergence in styles and anthers. Here, both antagonistic and coevolutionary processes can theoretically play roles in the formation of species. Below I discuss these processes in detail.

FROM ECOLOGICAL DIVERGENCE TO ECOLOGICAL SPECIATION

The concept of ecological speciation suggests that reproductive isolation is the result of ecologically driven divergence (Sobel *et al.*, 2010; Nosil, 2012). This requires that gene flow between the two divergent forms is prevented through the acquisition of reproductive isolation barriers (Coyne & Orr, 2004; Nosil, 2012; Sobel & Chen, 2014). Together with ecogeographic isolation (the extent at which geographic separation affects gene flow between taxa), floral isolation (a pre-pollination reproductive isolation barrier) appears to be more important than postzygotic isolation barriers in plants (Nosil, 2012), which highlights the perceived importance of pollinators in driving speciation. In the event of secondary contact between two diverged forms, partial reproductive isolation can occur if different forms are pollinated by different pollinators (Ramsey *et al.*, 2003; Kay, 2006). This is unlikely to generate full reproductive isolation because generally, pollinators are not completely species specific (Hopkins & Rausher, 2014; Campbell *et al.*, 2015). In contrast to the idea that pollinators are usually important isolating barriers in secondary contact zones, many cases of plant range extension involve the movement of plants into areas without the pollinators to which they are adapted. Consequently, secondary contact zones may not have the pollinators that drove the initial processes of ecological divergence, and it is highly likely that in such cases, introgression will occur and the differences between the two phenotypes will break down (Coyne & Orr, 2004). However, it is also possible that additional barriers may have accumulated as a non-adaptive byproduct of

ecological divergence (Coyne & Orr, 2004; Jewell *et al.*, 2012). For example, Jewel *et al* (2012) has shown that postzygotic isolation barriers were stronger and faster evolving than prezygotic isolation barriers including pollinator isolation in both allopatric and sympatric taxa of Chilean bellflowers (*Nolana*). Importantly, in recently diverged populations, most reproductive isolation barriers, including floral isolation are likely to be partial and may act in concert to lead to complete reproductive isolation (Ramsey *et al.*, 2003; Anderson *et al.*, 2016a).

In the *P. longipennis* system, specialization by pollinators in secondary contact zones may not be important for the evolution of reproductive isolation. For example, partial reproductive isolation may result if ecotypes with different tube or style lengths place and receive pollen on different parts of a pollinator's body. Differences in style length may contribute to reproductive isolation for *N. humilis* if ecotypes were to make secondary contact. This is evidenced by the observation that reproductive parts of introduced flowers seldom make contact with the bodies of local pollinators, whereas the reproductive parts of the local forms do (Newman *et al.*, 2015). Similarly, character displacement may also generate style length differences between populations which could act in concert with trait matching as gene flow barriers upon secondary contact. Many of the floral ecotypes also appear to exhibit differences in flowering time (Newman, unpublished) and these differences are possibly adaptations to the phenophase of different pollinator species. If these differences are maintained in sympatry, this could result in a very important geneflow barrier. Anderson *et al* (2010a) found that flowering differences were the most important geneflow barrier for short and long tubed forms of the hawkmoth pollinated *Gladiolus longicollis*. Incompatibilities between recently diverged forms may also contribute to reproductive isolation in the absence of different kinds of pollinator. For

example, I found that ecotypes of *P. dipetalum* which appeared to be adapted to long-proboscid flies versus short proboscid insects had evolved incompatibilities in allopatry (Newman unpublished). Anderson *et al* (2016) similarly found that in a secondary contact zone with just a single pollinator species, two tube length ecotypes of *Lapeirousia anceps* had also evolved incompatibilities.

Unlike the study of Anderson *et al* (2016), which found secondary contact zones of recently diverged ecotypes, I was unable to find any secondary contact zones for ecotypes in the systems that I studied for this thesis. One likely explanation for this may be that recently diverged populations seldom accumulate enough reproductive isolation and that differences are soon lost through introgression (Nosil, 2012). Another explanation could be that the distributions of different pollinators are linked to different habitats. For example, Johnson (2007) has shown that pollination ecotypes of the orchid *Satyrium hallacki* occur in different habitats which are linked to the reproductive requirements of their pollinators. Consequently, secondary contact zones are likely to be extremely rare or they may never occur. This highlights the possibility that speciation may occur without secondary contact ever being made. This is supported by species distribution patterns where sister species occupy different mountain ranges (Newman *et al.*, 2012). Furthermore, the incompatibilities between *P. dipetalum* pollination ecotypes with non-overlapping ranges also suggests that secondary contact is not necessarily an important part of the speciation process. Lastly, phylogenetic studies have recorded that habitat shifts are the strongest drivers of speciation in South Africa (Van der Niet & Johnson, 2009; Slingsby *et al.*, 2014; Verboom *et al.*, 2015). This may act synergistically with the distribution of pollinators to drive speciation (Johnson, 2010).

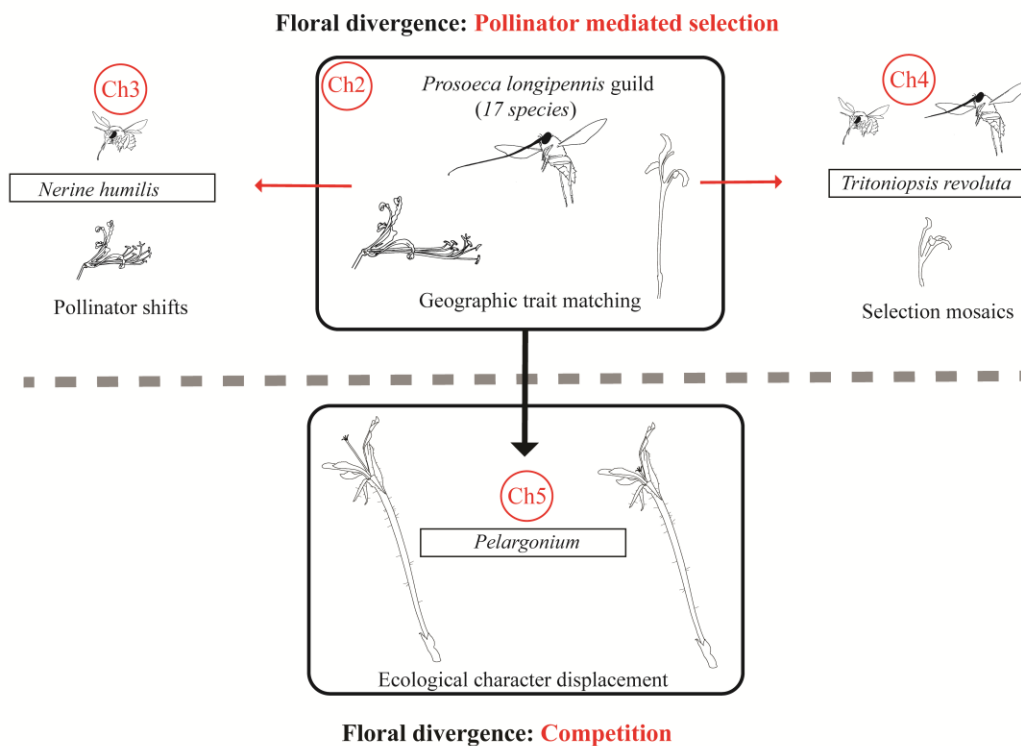


Fig. 6.1. The above figure illustrates the mechanisms generating floral variation within the *Prosoeca longipennis* system. Above the stippled line; floral divergence occurs mainly through adaptation to pollinator morphology. In chapter two, I have shown that floral divergence can evolve through geographic trait matching between corolla/style length and the morphology of the pollinator without invoking a pollinator shift. In chapter three, I have shown that when *Nerine humilis* occurs outside the range of the fly, floral divergence can arise through local pollinator adaptation via the mechanical fit between flower and pollinator morphology. In chapter four, *Tritoniopsis revoluta* is visited by more than one functional pollinator type. In this chapter; I show that the additive effects from multiple functional pollinator types, each contribute individually to overall fitness. In my final data chapter, chapter five (below the stippled line), I show how floral variation may arise in style and stamen exertion length as a result of ecological character displacement resulting from competition for pollen placement.

Taken together (Fig.6.1), this thesis presents evidence to suggest that pollinators are important in driving divergence among populations. Since several phylogenetic studies demonstrate that pollinators appear to be important in the speciation process, this thesis highlights the seamless continuity between microevolutionary processes and macroevolutionary patterns in plant speciation. Importantly, this thesis provides direct evidence for pollinator driven divergence on plant populations whereas the evidence suggested by phylogenetic studies are merely inferred. This thesis also demonstrates that character displacement may drive the divergence of populations. In contrast to phylogenetic studies of floral adaptation to different pollinators, there are only two phylogenetic studies that suggest that character displacement has an important role to play in the speciation process (Grossenbacher & Whittall, 2011; Koski & Ashman, 2016). I suggest that this paucity of evidence probably represents a gap in the literature, rather than the lack of importance for character displacement as a driver in the speciation process. Lastly, floral variation arising through adaptation to pollinators and character displacement should not be seen as mutually exclusive, as these processes may act synergistically in generating floral diversity. Despite the interaction between these processes being potentially important in driving floral divergence, this topic remains relatively unexplored and provides an important avenue for future research.



Photo: Genevieve Theron

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