UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Investigating environmental variables that facilitate changes in flower colour morphs in the Drakensberg *Rhodohypoxis baurii*

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for my beloved mother, Rifumuni Maria Mtileni, for everything.
Thank you with everything I am.
I love you.

"We will have eternity to celebrate victories
but only a few hours before sunset to win them."
Amy Carmichael

DECLARATION

I declare that this dissertation is my own, unaided work, unless stated otherwise. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University or similar institution.

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PREFACE

The dissertation comprises an introductory chapter (Chapter One), two data chapters (Chapters Two and Three) and a concluding synthesis chapter (Chapter Four). The two data chapters are written up as manuscripts, so there is some repetition between them. The last chapter provides the general discussion of the study's findings in a broader context of flower colour evolution, future research directions and concluding remarks.

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ABSTRACT

The maintenance of diversity in floral traits and flower colour polymorphism (FCP) have been of interest in evolutionary biology. As such, it is becoming increasingly necessary to examine the ecological and evolutionary explanations of FCPs in populations or species, especially since a growing body of work on flower colour continues to form the basis of important discoveries in biology. Specifically, environmental variables affect FCPs within populations or species, thereby facilitating speciation and subsequent floral evolution.

The near-endemic Drakensberg plant species, *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt is a non-model system that occurs in a vulnerable mountain grasslands habitat and comprises populations with FCPs. Investigating how intraspecific FCP (within taxon and population) relates to environmental variables and subsequent fitness across the flowering season in *R. baurii* var. *confecta* may enable us to make accurate predictions about the evolutionary trajectories of naturally polymorphic populations and provide insight into how this species may respond to environmental change.

I attempted to replicate field conditions where soil moisture and solar radiation differ at three time points (early, mid- and late flowering season) at a polymorphic population of *R. baurii* var. *confecta* that occurs in Sentinel Peak car park (SCP), Free State, South Africa, in a growth chamber experiment, to investigate whether soil moisture or solar radiation, or their interactive effect underpin the shift in flower colour morphs (from a majority of white flowers to a majority of pink flowers as the flowering season progresses). My findings indicated that solar radiation, but not soil moisture best explained the shift in white and pink flowers over the flowering season. Specifically, there were more pink than white flowers when both soil moisture and solar radiation were relatively higher towards the end of the

flowering season than in the early and mid-time points over the flowering season, when flowers are predominantly white. In the growth chamber experiment, I used UV exposure as a proxy for increased solar radiation. Only combinations of well-watered and UV exposure had a significant overall effect on the percentage of pigmented flowers produced during the experiment.

Further, I performed an amplified fragment length polymorphism (AFLP) scan in three populations of *R. baurii* var. *confecta* with different extents of FCPs (the SCP population and two monomorphic populations) at a single locality in Free State, South Africa, to compare among-population genetic differentiation. Interestingly, only one outlier locus was detected in the *R. baurii* var. *confecta* genome scan but there was little support that it was effective. The outlier locus was found in 80% and 30% of individuals from the flat catchment and Witsieshoek lodge populations (the monomorphic populations), respectively, while only 8% of individuals in the polymorphic SCP population possessed the outlier locus. However, no genetic differences among populations were significant, with little molecular variance among populations (3%). Together, my findings highlight that FCP in *R. baurii* var. *confecta* may be due to population response to environmental change, but not population genetic differences. Consequently, FCPs in this study system are likely population-specific. Nevertheless, my research contributes to the growing body of work that investigates the relationship between environmental variables and FCPs, and offers insights into the ecological and genetic relationship with regards to FCPs and how they are maintained in a mountain endemic plant.

GLOSSARY OF ABBREVIATIONS

AFLP: Amplified fragment length polymorphisms

ABP: Anthocyanin biosynthesis pathway

AMOVA: Analysis of molecular variance

Bp: Base pairs

CTAB: Cetyltrimethylammonium bromide' method of DNA extraction.

CAT: Central Analytical Facility

DHK: Dihydrokaempferol

DHM: Dihydromyricetin

DHQ: Dihydroquercetin

FCP: Flower colour polymorphism

FDR: False discovery rate

Lmer: Linear mixed-effects regression

M a.s.l: Meters above sea level

NGS: Next-generation sequencing

PO: Posterior odds

PCoA: Principal coordinate analysis

RJMCMC: Reversible-jump Monte Carlo Markov chain

SCP: Sentinel Peak car park

SNP: Single nucleotide polymorphism

qPCR: Quantitative polymerase chain reaction

UV: Ultraviolet light

UV-VIS: Ultraviolet-visible

VSWC: Volumetric soil water content

UV-/W+: Well-watered conditions with no UV treatment

UV+/W+: Well-watered conditions and UV exposure treatment

UV-/W-: Water-deficit conditions with no UV treatment

UV+/W-: Water-deficit conditions and UV exposure treatment

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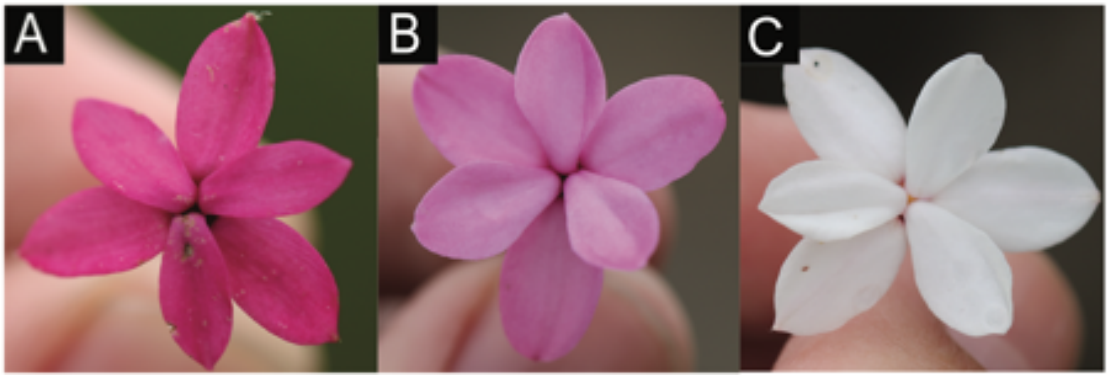
General Introduction



Flower colour morphs of the study system, *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt: bright pink (A); pale pink (B); and white (C). Extracted with permission from Gardiner & Glennon (2019)

1.1 Introduction & background information

A growing body of work on flower colour continues to form the basis of important discoveries in biology (e.g., Wright 1943; Schemske & Bierzychudek 2001; Strauss & Whittall 2006, Ng & Smith 2018), specifically, the maintenance of diversity in floral traits and flower colour polymorphism (FCP hereafter) have been of interest in evolutionary biology (e.g., Kay 1978, Wright 1978; Rausher 2008; McKinnon & Pierotti 2010; Sobel & Streisfeld 2013; Carlson & Holsinger 2015). Plants may exhibit trait variation (e.g., Picotte et al. 2007), like FCPs within a population (Schiestl & Johnson 2013), in response to environmental change such as changing levels of temperature or water stress, to increase survival and fitness (e.g., Schemske & Bierzychudek 2001; Strauss & Whittal 2006; Arista et al. 2013). As such, it is becoming increasingly necessary to examine the ecological and evolutionary explanations of FCPs in populations or species.

In the same plant population, there can be distinct variants of flower colours (which I will term 'morphs' per Narbona et al. 2018; for an example see Fig. 1.1). Narbona et al. (2018) reported that two or more morphs should coexist in at least one population for a species to be considered polymorphic (e.g., white, light and dark pink morphs of *Silene littorea* Brot.; Casimiro-Soriquer 2016) and the authors suggest that the variation in morphs is unlikely due to recurring mutations (Ford 1945; Huxley 1955). Species may be monomorphic in some populations and show FCP in others (Wright 1978; McLean & Stuart-Fox 2014; Forsman 2016). In some instances, a single species may comprise varying frequencies of different morphs in populations through time (e.g., *Linanthus parryae*; Schemske & Bierzychudek 2001).

Although FCPs may have a genetic underpinning, it is possible that phenotypic plasticity may cause FCP as alternative morphs may also be due to plant response to environmental cues (e.g., *Silene littorea*; Del Valle et al. 2018a). FCPs may also result from a

random switch between phenotypes (reviewed in Wennersten & Forsman 2012). However, the source of FCP does not affect the capacity of polymorphic populations to persist in changing environments compared to monomorphic populations (Wennersten & Forsman 2012). Nevertheless, natural selection may be due to the variability resulting from genetic underpinning of FCPs as there may be a positive relationship between alternate morphs and increased adaptive potential (Forsman et al. 2008). My research considers both the genetic basis of FCPs and environmental variables that relate, directly, to FCP-maintenance, to better understand how and why the FCP is maintained.

Little is known about why FCPs are maintained in a population (Narbona et al. 2018) or how ecological factors lead to evolutionary consequences of FCPs (Strauss & Whittall 2006). FCPs may play a role in driving speciation by promoting reproductive isolation (pre- and postzygotic reproductive isolating barriers that prevent gene flow; Lowry et al. 2008) and subsequent selection (Servedio et al. 2011). Specifically, the type of variability resulting from FCPs may form the basis of floral evolution (Schemske & Bradshaw 1999; Fenster et al. 2004; Schiestl & Johnson 2013) as pollinators might target one morph over the other (e.g., Schemske & Bradshaw 1999; Gómez 2000). In addition, selection related to environmental variables and environmental heterogeneity (potential selection agents) may maintain FCPs (Warren & Mackenzie 2001).

More research is needed to test ecological and evolutionary hypotheses with regard to FCPs and how they occur and are maintained in non-model species (but see Hopkins & Rausher 2011; Hopkins et al. 2012), especially in species that may be exposed to relatively harsh environmental conditions such as low soil moisture, direct sunlight, and freezing temperatures even during summer months (for an example see study system; *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt).

Fig. 1.1 Examples of species with flower colour polymorphism in the Mediterranean Basic. A) Purple and yellow-flowered plants of *Iris lutescens* Lam. (Iridaceae), Montpellier, France. B) Yellow, orange, pink and white-flowered plants of *Helianthemum nummularium* (L.) Mill. (Cistaceae), Ormea, Italy. C, D, E) Three colour morphs of *Lavatera trimestris* L. (Malvaceae), Seville, Spain. F, J, Blue and orange-flowered plants of *Lysimachia arvensis* (L.) U. Manns & Anderb (Myrsinaceae), Sevilla, Spain. G, H, I) Three colour morphs of *Silene littorea* Brot. (Caryophyllaceae), Pontevedra, Spain. K, L) Pink and white-flowered plants of *Orchis italica* Poir. (Orchidaceae), Seville, Spain. M) Red and yellow-flowered plants of *Adonis microcarpa* DC. (Ranunculaceae), north Morocco. Extracted with permission from Narbona et al. (2018).

1.2 Anthocyanins & their implications for FCP evolution

The majority of known floral pigments are made of three groups of compounds - betalains, carotenoids and anthocyanins (Grotewold 2006), however, a wide range of floral colours (most of the bright red, pink and blue colours) are produced from the synthesis of anthocyanins (Dooner & Robbins 1991; Holton & Cornish 1995). Specifically, unpigmented morphs may lack anthocyanin content when compared to pigmented morphs (e.g., *Silene littorea* Brot.; Casimiro-Soriquer 2016).

Anthocyanins are a clearly visible class of secondary plant metabolites (flavonoids; Dooner & Robbins 1991; Holton & Cornish 1995) that may be compartmentalized in various tissues of the plant, primarily leaves, flowers and seeds, or exclusively in roots and shoots (Chalker-Scott 1999). Anthocyanins are the most common and water-soluble floral pigments and are produced by the anthocyanin biosynthesis pathway (ABP; Rausher 2008). These pigments are the most conserved among angiosperms (Holton & Cornish 1995; Winkel-Shirley 2002). For example, 87.7% of plant species that occur in the Mediterranean Basin have FCPs that are based on anthocyanins (Narbona et al. 2018).

The ABP is a pleiotropic pathway where at least eight specific genes work together to produce anthocyanins (Dooner & Robbins 1991; Grotewold 2006). Specifically, three large gene families (R2R3-MYB, basic helix-loop-helix and WD40-repeat) encode proteins that make up the transcription complex (Ramsay & Glover 2005), which regulates ABP and controls other pathways that affect the production of root hairs and formation of trichomes (may assist in water use efficiency; e.g., Picotte et al. 2007; Hamaoka et al. 2017), among other aspects of epidermal cell fate (Ramsay & Glover 2005). Traits resulting from the ABP can be expected to be beneficial and adaptive as the pathway is highly conserved across flowering plants (Holton & Cornish 1995; Winkel-Shirley 2002). In general, selection should favour ABP-produced traits, like FCPs, as they may increase plant ability to persist and

succeed in the face of environmental change (i.e., ecological and evolutionary success of populations and species; Forsman 2016).

1.3 Selection agents that affect the presence/absence of FCPs

Selection pressure exerted by pollinators can facilitate the diversity of floral traits (e.g., Fenster et al. 2004; Schiestl & Johnson 2013) as pollinators might target one morph over the other (e.g., Schemske & Bradshaw 1999). For example, pollinators prefer unpigmented (white) over pigmented (purple) morphs in *Lobularia maritima* (Gómez 2000). Further, pollinators show a differential preference of morphs in two closely related species of monkeyflowers, *Mimulus lewisii and M. cardinalis*; bees prefer flowers with low anthocyanin content whereas hummingbirds prefer high anthocyanin content (Schemske & Bradshaw 1999). Such results suggest that pollinator preference and subsequent FCPs contribute to reproductive isolation between related taxa, subsequent divergent selection (Servedio et al. 2011) and floral evolution (Schemske & Bradshaw 1999; Fenster et al. 2004; Schiestl & Johnson 2013). Pollinator preference for rare morphs may result in negative-frequency dependent selection where the frequency of morphs decrease as they become common (e.g., rewardless orchid *Dactylorhiza sambucina* (L.); Gigord et al. 2001) or positive-frequency dependent selection where the frequency of morphs increase as they become common (e.g., foraging bias of bumblebees *Bombus terrestris* (L.) to conspicuous blue flowers; Smithson & Macnair 1996).

In some instances, pollinator preference is not observed – pollinators visit all morphs equally (e.g., *Iris lutescens* Lam.; Imbert et al. 2014a) and do not discriminate between morphs (Miller 1981). In other systems, observations at the study site have not revealed any pollinators - e.g., *Mimulus verbenaceae* in Vickery (2008) and *Mimulus luteus* in Carvallo & Medel (2010). Such examples indicate that selection by pollinators may not contribute greatly

to the maintenance of FCPs in all species when compared to other potential selection agents (for an example see study system; *Rhodohypoxis baurii* (Baker) Nel. var. *confecta*).

Outside of pollinator preference, or lack of thereof, floral morphs may have a differential tolerance to abiotic conditions (Levin & Brack 1995; Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008; Arista et al. 2013). Arista et al. (2013) reported that orange morphs of the scarlet pimpernel (*Lysimachia arvensis* (L.) U. Manns and Anderb. perform relatively better in wet and sunny environments whereas blue-flowered plants are more tolerant to water-stressed environments. In general, pigmented morphs may be favoured in drought and hot conditions than unpigmented morphs (Warren & Mackenzie 2001; Schemske & Bierzychudek 2001, 2007; Strauss & Whittall 2006; Arista et al. 2013).

Plants that can regulate the expression of anthocyanins in vegetative tissues may benefit from an increased adaptive potential in response to environmental pressures (Dick et al. 2011). This is because anthocyanins may enhance tolerance to heat, cold temperatures and water-deficit conditions, among other environmental conditions (Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008). In high light conditions, anthocyanins may absorb parts of UVA and UVB spectra, thereby providing effective sunblock against solar radiation (UV-screening; Takahashi & Badger 2011), to avoid damage to the photosynthetic machinery and subsequent photoinhibition (Takahashi & Badger 2011). In addition, anthocyanins may be induced by osmotic stress and have been reported to be drought resistant (Chalker-Scott 1999).

Anthocyanin production may be costly in environments that are not stressful (Warren & Mackenzie 2001). Consequently, one would expect to observe pigmented morphs in stressful environments such as mountain grasslands or alpine environments; these systems occur worldwide and there is an extreme variability regarding water availability, altitude and

seasonality (Grabherr et al. 2010). Seasonality, altitude (air density), and water availability determine ecosystem structure and function in mountain systems (Grabherr et al. 2010). For example, in response to seasonal climates and long winters, alpine plants may be frost resistant or become dormant to survive under snow protection (Grabherr et al. 2010). Interestingly, due to attitudinal variations in rainfall, temperature and solar radiation, pigmented morphs may be favoured at higher elevations (e.g., pink morphs of South African *Protea*; Carlson & Holsinger 2015). As such, flower colour can be viewed as a proxy for a plant responding to environmental change, and environmental variables may be important selection agents that facilitate floral evolution.

A balance between migration, genetic drift and mutation may alternatively cause FCP persistence (Narbona et al. 2018) or loss of anthocyanins (e.g., Twyford et al. 2018). FCPs may be consistent with natural selection (Schemske & Bierzychudek 2001, 2007) or genetic drift (Wright 1943, 1978; Lack & Kay 1988). However, most studies support that natural selection plays a dominant role in FCP maintenance (e.g., Schemske & Bierzychudek 2001; Hopkins et al. 2012). In a mutant population of *Mimulus guttatus*, the loss of anthocyanin expression is associated with a decrease in the expression of transcription factors (i.e., *MYB5*) that regulate anthocyanin pigmentation due to selection pressure in the wild (Twyford et al. 2018). Nevertheless, more research is needed to investigate the role of genetic drift and natural selection on flower colour divergence (Schemske & Bierzychudek 2007; Rausher 2008) and the complex interplay of factors that may affect FCP maintenance in nature (Twyford et al. 2018).

Here, I focused mainly on FCP as it relates to the cost-benefit trade-off of anthocyanin production in relation to environmental variables. As observations in the field have not revealed obvious pollinators that may be pollinating *R. baurii* var*. confecta*, it is possible that FCP may not be attributable to pollinator-mediated selection in this study

system. Also, we do not know about the heritability of flower colour, genetic basis or plasticity of FCP in this system. Future work aims to focus on a formal quantitative study, a study to assess multiple generations of flower colour morphs in the greenhouse, and to test the effect of migration, genetic drift and mutation on FCP. My research forms the basis for future work in the lab, to gain an in-depth understanding of FCP in this system.

1.4 FCPs & fitness

FCPs are frequently associated with plant fitness (e.g., seed production, seedling survival and number of flowers) due to abiotic factors (Schemske & Bierzychudek 2001; Strauss & Whittal 2006; Arista et al. 2013). For example, pigmented morphs (blue) may have a fitness advantage relative to unpigmented morphs (white) in years of low spring precipitation (e.g., *Linanthus parryae*; Schemske & Bierzychudek 2001) and blue morphs may perform better in xeric environments (Arista et al. 2013). In some instances, unpigmented morphs may not demonstrate fitness costs relative to pigmented morphs (e.g., anthocyanin-deficient genotype of *Mimulus guttatus*; Twyford et al. 2018). It is necessary to investigate fitness to flower colour as some morphs may be more fit than others, and potentially under selection in nature.

Polymorphic populations (regardless of the source of variation- e.g., phenotypic plasticity and genetic polymorphism) may be less vulnerable to environmental change compared to monomorphic populations (Forsman et al. 2008; Wennersten & Forsman 2012). This is because FCPs may reduce intraspecific competition and promote niche differentiation among morphs (differential utilization of resources), enhance colonization success, distributional range expansions, population persistence and stability, thus, speciation and floral evolution (Forsman et al. 2008).

Fitness differences (whether direct or indirect) of alternative colour morphs can be complicated (Forsman 2016). For instance, an increase, or decrease, in the adaptive potential

and long-term fitness of populations or species may result from colour transitions (e.g., from poly- to monomorphism) due to natural selection, irrespective of the differential fitness effects of colour morphs (Forsman 2016). Such a decrease or increase in adaptive potential and fitness may occur because evolution by natural selection is a non-random process with no predetermined goal, which might explain why FCPs are not more widespread across species ranges (Forsman 2016). In addition, the consequences and ways that FCPs may affect the ecological and evolutionary success (e.g., population stability) of populations or species differ from the mechanisms and causes (e.g., selection agents) that affect the absence/presence of FCPs and how they are maintained in natural populations (Forsman 2016). More research is needed to investigate FCP effects on the fitness of individuals, alternative colour morphs, populations and species. Linking FCPs and fitness may provide a clearer understanding of why FCP is maintained in natural populations.

1.5 Molecular techniques to examine the basis of FCPs

In exploring floral evolution and the speciation process, genetic methods, like next-generation sequencing (NGS), gene expression with quantitative PCR (qPCR) and transcriptomics can help examine what molecular construct underpins FCPs. For example, if we know the ABP structural genes and their *trans*-acting transcription factors (e.g., *MYB5*), we may better understand the subsequent formation of anthocyanin pigmentation and FCP in single or multiple populations. Using such information, we can then link FCP maintenance in a population to ecological factors that may initiate evolutionary effects of FCPs.

Although qPCR (quantitative PCR) is a relatively rapid and cost-effective approach in quantifying gene expression, it has been associated with presenting discordant and contradictory data. The technique has been generally reported as being inconsistent, complex and inadequately standardised (Bustin 2010). Similarly, a mRNA-based approach (NGS transcriptomics) may also present challenges as sufficient quantity RNA is necessary for this

method and it is more susceptible to extraction problems compared to a straightforward DNA-based approach (NGS). Nevertheless, transcriptomics presents a useful and informative opportunity and powerful method to study the expression of specific structural genes, especially for genomes that are comparatively large and complex (Davidson et al. 2011).

Several population genetic-based approaches, like amplified fragment length polymorphisms (AFLPs) and single nucleotide polymorphisms (SNPs), have also been developed for genotyping large populations, although SNPs have been used in model systems (e.g., Fournier-Level et al. 2011). AFLP, a PCR-based and commonly used technique in ecology and population genetics (Mueller et al. 1999) is a useful tool that can be applied for genome-wide detection of genetic polymorphisms on almost all organisms (Bensch & Akesson 2005; Meudt & Clarke 2007; Vuylsteke et al. 2007). The AFLP technique uses restriction enzymes to digest genomic DNA, and has been reported as being robust and repeatable, with the capacity to generate large amounts of allele frequency data (Mueller et al. 1999). Here, I used a plant AFLP procedure per Blignaut et al. (2013) to compare AFLP-based genetic differences among three *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt populations where one population experiences a marked shift in white and pink morphs and two populations do not (Chapter Three).

1.6 Rationale

Most studies on FCPs have mainly focused on model systems or plant species in the Mediterranean (e.g., Narbona et al. 2018), with a few studies on non-model systems (but see Hopkins & Rausher 2011; Hopkins et al. 2012). *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt is a non-model system that occurs in a vulnerable mountain grasslands habitat (see O'Connor 2005) and comprises populations with FCPs. Investigating how intraspecific FCP (within taxon and population) relates to environmental variables and subsequent fitness across the flowering season in *R. baurii* var. *confecta* may enable us to

make accurate predictions about the evolutionary trajectories of naturally polymorphic populations and provide insight into how this species may respond to increasingly harsh environmental conditions (e.g., increased solar radiation) over time.

This study aimed to contribute to a growing body of work that investigates the relationship between FCP and potential environmental correlates (e.g., Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Narbona et al. 2018) and compares fitness between morphs (Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Arista et al. 2013). I acknowledge that there is a complex interaction of many factors in maintaining FCPs in natural populations (e.g., mutation and genetic drift) and have attempted to reduce confounding variables through controlled experiments. As such, my research focuses on two environmental variables (soil moisture and solar radiation/UV exposure) that may facilitate changes in flower colour morphs, whether there may be genetic differences in populations with clear shifts in colour morphs through the flowering season, and if colour morphs exhibit a difference in fitness (e.g., seed set and germination) in *R. baurii* var. *confecta*. Such knowledge is useful when trying to assess how FCPs relate to floral diversity within a South African context or similar contexts. Identifying the effect of environmental variables on FCP within populations and subsequent fitness effects, and potential population genetic differences provides an opportunity to better understand floral evolution. Specifically, it is necessary to investigate and understand environmental variables that may affect the presence/absence of FCPs, and how floral diversity may change in response to environmental change. Environmental variables affect FCPs within plant populations or species, thereby facilitating speciation and subsequent floral evolution. If FCPs are present in a population and selection acts strongly on them, this may lead to potential genetic divergence, which may lead to adaptation and increased floral diversity. Such research is more important for endemic and near-endemic plant species that may be found in vulnerable habitats that may be

vulnerable to global change, like *R. baurii*; these plants may adapt to environmental change through the variability in traits, like FCP.

1.7 Study system

The South African near-endemic Drakensberg plant genus, *Rhodohypoxis* Nel. (Hypoxidaceae) shows FCPs both across taxa and within specific populations. The existence of this genus is potentially threatened, as the mountain grasslands where it occurs are vulnerable to global change and transformation (e.g., O'Connor 2005). *Rhodohypoxis* species are herbaceous and geophytic perennials that exist primarily throughout the Drakensberg, with some populations reaching into the KwaZulu-Natal Midlands (Hilliard & Burtt 1978). This small genus comprises six species, among which *R. baurii* (Baker) Nel. shows FCP. *Rhodohypoxis baurii* can be distinguished by the formation of colourful carpets of white, pink and red flowers (Hilliard & Burtt 1978). The distribution of *R. baurii* ranges between Barberton (Mpumalanga) and Mthata (Eastern Cape), above 1100 m a.s.l, with the center of the distribution ranging along the KwaZulu-Natal Drakensberg mountain grasslands and into Lesotho (Hilliard & Burtt 1978).

Hillard & Burtt (1978) describe three *R. baurii* varieties using both morphological and ecological traits (Table 1.1). Briefly, *R. baurii* var. *baurii* occurs on damp, partly shaded cliff faces or rock flushes and comprises predominantly deep red flowers. *Rhodohypoxis baurii* var. *platypetala* grows on dry, shallow, stony soils over rock sheets and comprises predominantly white flowers.

Table 1.1 Summary of Hilliard & Burtt's (1978) description of habitats and vegetative traits for the three varieties. Please note that *Rhodohypoxis baurii* var. *platypetala* and *Rhodohypoxis baurii* var. *baurii* are for reference purposes only and were not used in the study.

Rhodohypoxis baurii variety	Habitat description	Leaf and floral descriptions
var. *baurii*	Seasonally moist rocky habitats (e.g., damp partly shaded cliff faces or rock flushes)	Leaves are long, erect, and narrow. Flowers are predominantly deep red.
var. *platypetala*	Dry, shallow, stony soils over rocky sheets	Leaves are short, broad, and mostly on the ground. Flowers are predominantly white.
var. *confecta*	Grassy rock slopes at high altitudes above 2600 m a.s.l.	Leaves are long, erect, and narrow. Flowers are white, pale pink, or bright pink.

The variety that was the focus of this study is *R. baurii* (Baker) Nel. var. *confecta* and it grows on damp, grassy slopes that consist of partly shaded rock flushes or cliff faces and FCPs are present in populations. *Rhodohypoxis baurii* var. *confecta* has the most striking FCPs – sheets of mixed white, pink and red-flowered plants (Hilliard & Burtt 1978). Leaves are erect and bright green, with leaf length and width averaging 25-75 and 1.5-5 mm, respectively (Hilliard & Burtt 1978). Hilliard & Burtt (1978) stated that flowers open white and age through pink to red; however, preliminary data suggest this is not the case. *Rhodohypoxis baurii* var. *confecta* is a high-altitude plant, occurring at 2550 m a.s.l on mountain grasslands (Hilliard & Burtt 1978).

Rhodohypoxis baurii var. *confecta* flowers are white, pale pink or occasionally bright pink (three distinct colour morphs within a population; Fig. 1.2, 1.3). When produced, bright pink flowers are usually only observed for a handful of individuals (pers. obs). In general, *R. baurii* var. *confecta* flowers emerge from buds within two or three days to become fully open and senesce after approximately 14 days. No pollinators have been observed visiting these flowers and artificial pollination does not induce flower colour change either (pers. obs). In

addition, preliminary data suggest that the three varieties are self-compatible (unpublished data). At the Sentinel Peak car park (SCP) population in the Free State province of South Africa, *R. baurii* var *confecta* individuals occur at 2550 m a.s.l and experience high solar radiation, low water availability, and large variations in temperatures during summer months when flowers are open (ranges 5-35ºC; Gardiner & Glennon 2019).

Fig. 1.2 White, pale and bright pink morphs of *Rhodohypoxis baurii* (Baker) Nel. var. c*onfecta* Hilliard & Burtt (Photo: K. Glennon).

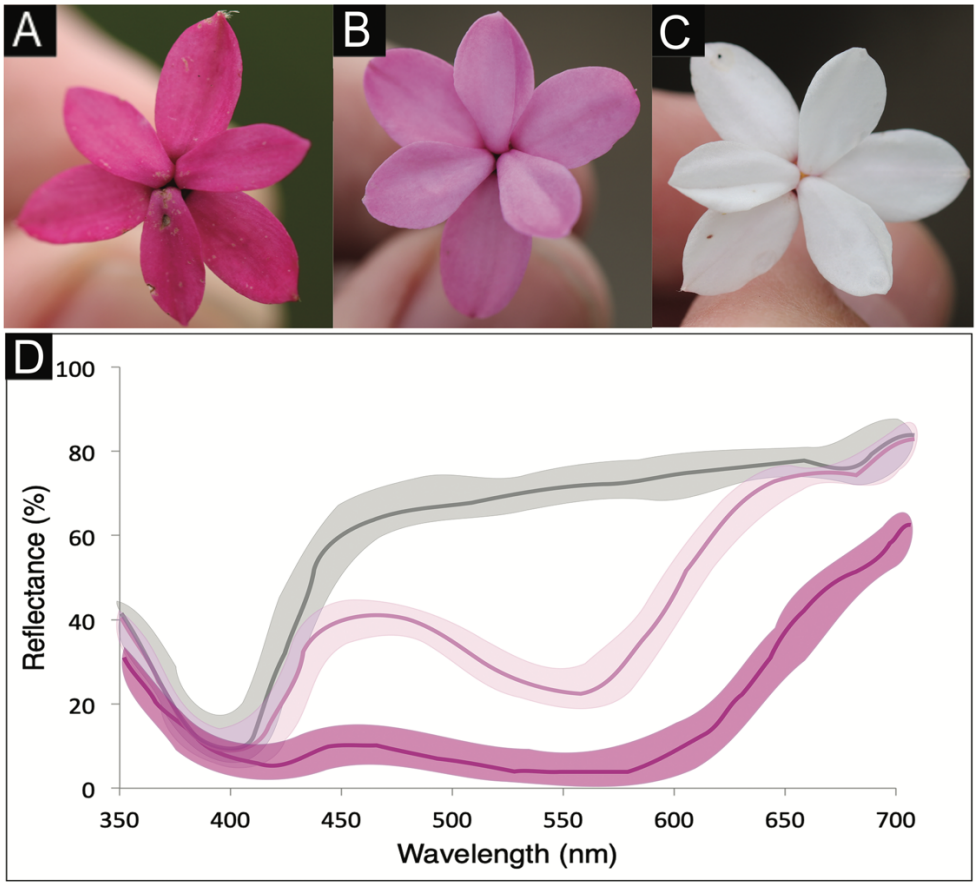


Fig. 1.3 A, B, C) Flower colour morphs of *Rhodohypoxis baurii* (Baker) Nel. var. *confecta*: bright pink (A); pale pink (B); and white (C). D) Averaged ultraviolet-visible (UV-VIS) spectral data for the upper petal surface of white (grey line), pale pink (light pink line) and bright pink flowers (dark pink line). Standard errors are shaded around the lines. Extracted with permission from Gardiner & Glennon (2019).

For this study, I focused on three populations of *R. baurii* var. *confecta* that occur at a single locality in Free State, South Africa (Fig 1.4; Hilliard & Burtt 1978). The three populations occur within 4.62 km of each other. The first population occurs near the SCP in the northern Drakensberg. At this site, *R. baurii* var. *confecta* is widespread both above and below the SCP hiking trail at 2550 m a.s.l. The population comprises thousands of *R. baurii* var. *confecta* plants with morphs ranging from white, pale pink and bright pink over the flowering season (October-January). In addition, plants are exposed to relatively low soil moisture, direct sunlight, and freezing temperatures even during summer months. Plants are exposed to direct sunlight from mid-morning to sunset (10h00-18h00), as the surrounding mountain cliffs provide some shading in the early morning. Previous work suggests that soil moisture is associated with a shift in flower colour morphs from a majority of white to a majority of pink morphs over the flowering season (Gardiner & Glennon 2019).

The other two populations, one located on a flat catchment at 2249 m a.s.l and the other behind Witsieshoek lodge at 2195 m a.s.l, are exposed to direct sunlight until mid-afternoon, when the sun is below the escarpment. Interestingly, these two populations do not experience a marked shift in white and pink morphs (flowers remain predominantly white over the flowering season) compared to the SCP population (Gardiner & Glennon 2019).

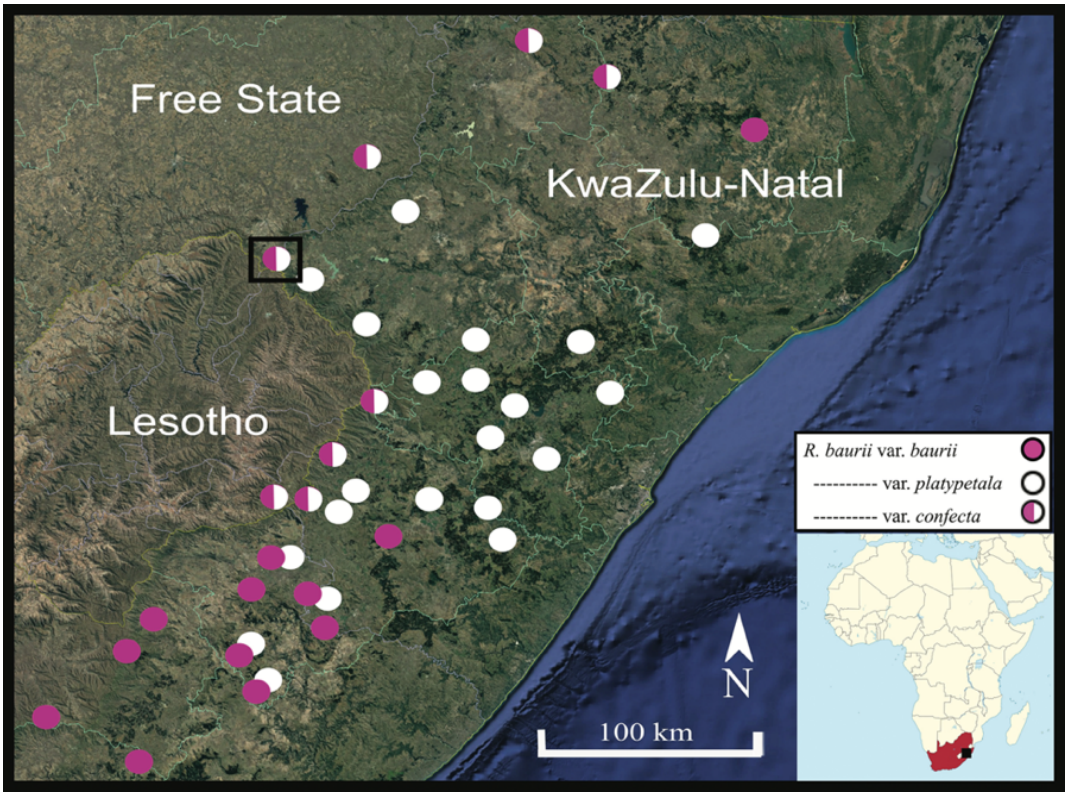


Fig. 1.4 Distribution of *Rhodohypoxis baurii* var. *confecta*, *Rhodohypoxis baurii* var. *baurii* and *Rhodohypoxis baurii* var. *platypetala* at a single locality in the Free State province, South Africa, and a sample location (noted with a square) of *Rhodohypoxis baurii* var. *confecta* used for this study. Extracted with permission from Gardiner & Glennon (2019).

1.8 Research aim & objectives

Although my research reflects a test of natural selection, I specifically investigated whether changes in solar radiation and soil moisture (outside of pollinator preference) may explain the shift from a majority of white flowers to a majority of pink flowers over the flowering season and compared genetic differentiation patterns between populations of the near-endemic Drakensberg plant, *R. baurii* (Baker) Nel. var. *confecta* with different extents of FCPs to attempt to relate the ecological and genetic relationship between my findings. This study aimed to investigate fitness differences between white and pink flower colour morphs, whether changes in soil moisture and solar radiation (UV) facilitate changes in morphs, and to investigate whether there are population genetic differences between polymorphic and

monomorphic *R. baurii* var. *confecta* populations. This aim was achieved through the following objectives (Fig. 1.5):

1.8.1 Objective 1: Establish whether fitness (e.g., flower number and seed set) differed between white and pink colour morphs within the same population (Chapter Two). The following question was addressed:

1) Are there fitness differences between the flower colour morphs both between plants observed in the field and between plants under controlled combinations of water-deficit and UV exposure conditions?

   Hypothesis: Pink morphs will be more fit than white morphs under UV exposure and increased soil moisture conditions, both in the field and in a controlled experiment.

1.8.2 Objective 2: Examine whether exposure to solar radiation (UV) and different soil moisture facilitated a shift in flower colour morphs (from a majority of white morphs to a majority of pink morphs) (Chapter Two). The following specific question was addressed:

1) Is there a relationship between the flower colours that emerge and the soil moisture and solar radiation individuals were exposed to?

   Hypothesis: Flower colour morphs will shift from a majority of unpigmented (white) morphs to a majority of pigmented (pink) morphs under increased soil moisture and solar radiation (UV) exposure.

1.8.3 Objective 3: To compare genetic differences between three populations where one population experiences a marked shift in white and pink morphs and two populations do not (Chapter Three). The following specific question was addressed:

1) Are there genetic differences between populations that may be linked to a change in pink and white colour morphs and those that do not?

Hypothesis: Individuals from the polymorphic population will comprise outlier loci that could be associated with changes in flower colour morphs and potential environmental variables.

1.9 Structure of the dissertation

The dissertation comprises an introductory chapter (Chapter One), two data chapters (Chapters Two and Three) and a concluding synthesis chapter (Chapter Four). The two data chapters are written up as manuscripts, so there is some repetition between them. The last chapter provides the general discussion of the study's findings in a broader context of flower colour evolution, future research directions and concluding remarks.

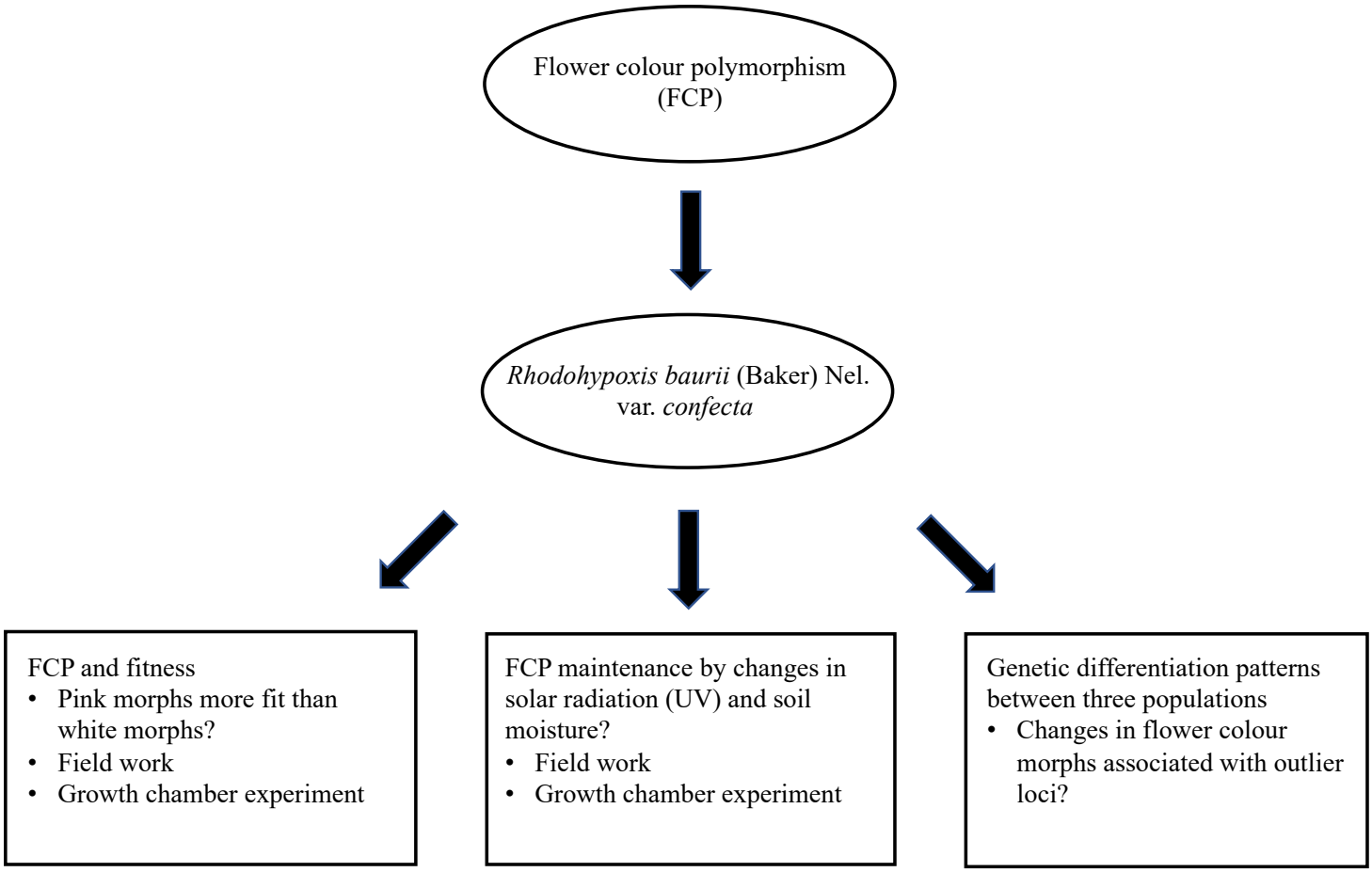


Fig. 1.5 An overview of the research objectives of this study. Overall research theme, flower colour polymorphism (FCP), and study system, *Rhodohypoxis baurii* var. *confecta*, are in oval boxes, with objectives in rectangular boxes.

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CHAPTER TWO

Increased solar radiation over the flowering season may explain the shift in flower colour morphs in a mountain endemic plant population

Sentinel Peak in the Free State province, South Africa, where a polymorphic population of *Rhodohypoxis baurii* (Baker) Nel. var. c*onfecta* Hilliard & Burtt occurs (Photo: K. Glennon)

2.1 Abstract

Environmental variables affect flower colour polymorphisms (FCPs) within plant populations or species, thereby facilitating speciation and subsequent floral evolution. Here, I attempted to replicate field conditions where soil moisture and solar radiation differ at three time points (early, mid- and late flowering season), in a growth chamber experiment, to investigate whether soil moisture or solar radiation, or their interactive effect underpin the shift in the proportion of white and pink morphs of the polymorphic Drakensberg near-endemic species, *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt. My findings from the field solar radiation, but not soil moisture best explained changes in the relative percentages of white and pink flowers over the flowering season. Specifically, there were more pink than white flowers when both soil moisture and solar radiation were relatively higher towards the end of the flowering season than in the early and mid-time points over the flowering season. To replicate field conditions in my growth chamber experiment, I used UV exposure as a proxy for increased solar radiation. Similarly, plants with exposure to UV and high soil moisture produced more pigmented flowers. My research contributes to the growing body of work that investigates the relationship between environmental variables and FCPs.

2.2 Keywords

Environmental variables, flower colour polymorphism**,** Hypoxidaceae, mountain grasslands endemic, solar radiation, soil moisture.

2.3 Introduction

Through the process of evolution, plants often modify morphological and physiological traits to increase survival and fitness in response to environmental variations such as changing levels of temperature and soil moisture (e.g., Picotte et al. 2007). Specifically, plants may exhibit trait variation through flower colour polymorphism (FCP hereafter) within a population and across species' ranges (Schiestl & Johnson 2013), however, little is known about how ecological factors lead to evolutionary consequences of FCPs (Strauss & Whittall 2006) or why FCPs are maintained in natural populations (Narbona et al. 2018).

In the same population of plants, there can be distinct variants of flower colour (termed 'morphs' per Narbona et al. 2018), with the variation coming from differences in flower colour expression and not due to recurring mutations (Ford 1945; Huxley 1955); Narbona et al. (2018) recently termed this flower colour polymorphism (FCP). In general, two or more morphs should coexist in at least one population for a species to be considered as colour polymorphic (Narbona et al. 2018). For example, FCP has been observed in the Iberian Peninsula endemic plant species, *Silene littorea* (Caryophyllaceae), where two out of 17 surveyed populations comprised three distinct colour morphs – white, light and dark pink morphs (Casimiro-Soriquer et al. 2016). In some instances, a single species may comprise varying frequencies of different morphs in populations through time (e.g., *Linanthus parryae*; Schemske & Bierzychudek 2001).

Flower colour is produced by the anthocyanin biosynthesis pathway (ABP), with anthocyanins being the most common pigments that produce flower colour (Holton & Cornish 1995; Grotewold 2006; Rausher 2008; Miller et al. 2011; Campanella et al. 2014). For example, Casimiro-Soriquer et al. (2016) found that petals of white-flowered morphs of *Silene littorea* lacked anthocyanin pigments while pigmented flower colours (light and dark

pink morphs) contained anthocyanin pigments. These floral pigments enable flowers to attract pollinators while maintaining their environmentally stress-related functions (Winkel-Shirley 2002; Dick et al. 2011). Nevertheless, although pollinators play a dominant role in the maintenance of FCP, a wide range of non-pollinator associated environmental variables have been reported to have an effect on FCP maintenance within populations or species, and the frequency of flower colour morphs in polymorphic populations (Strauss & Whittall 2006).

Outside of pollinator preference, anthocyanins play adaptive and protective roles in response to abiotic environmental stressors, and may enable plants to tolerate heat and water-deficit environmental conditions (Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008). As such, reduced soil moisture may be considered an important environmental stressor that plants respond to in their natural environments. In addition to unavailable soil water, changes in solar radiation may also be viewed as a potential environmental stressor to plants. Solar radiation induces the production of anthocyanins (Chalker-Scott 1999). Consequently, anthocyanins may provide an effective sunblock by absorbing parts of UVA and UVB spectra (Takahashi & Badger 2011). Subsequently, pigmented-flowered plants may be favoured over white-flowered plants in drought and heat environmental conditions because the ABP enhances stress tolerance via anthocyanin production (Schemske & Bierzychudek 2001, 2007; Strauss & Whittall 2006); pigmented morphs may be favoured in stressful environments than unpigmented morphs because of the benefits that the ABP provides (Schemske & Bierzychudek 2001; Strauss & Whittall 2006). Consequently, fluctuating environmental conditions may maintain FCPs, thus, pigmented and unpigmented morphs with a differential ability to tolerate environmental stressors (Dick et al. 2011). Further, FCPs are frequently associated with plant fitness (e.g., flower and seed production; Strauss & Whittall 2006). Consequently, flower colour can be viewed as a marker for a plant responding to environmental change. Therefore, identifying

environmental variables (potential selection agents) that potentially underpin FCPs within populations may provide an opportunity to better understand FCP maintenance and subsequent floral evolution.

The South African Drakensberg near-endemic plant genus, *Rhodohypoxis* Nel. (Hypoxidaceae) is a useful system to study whether shifts in the proportion of flower colour morphs (from a majority of white flowers to a majority of pink flowers over the flowering season) relate to environmental variables, and differences in fitness between the morphs. This genus comprises six species, among which the most widespread *Rhodohypoxis* species, *R. baurii* (Baker) Nel. shows FCP. In particular, one of the three *R. baurii* varieties, *R. baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt, shows FCP within a single population (three distinct morphs which are prevalent at different time points over the flowering season).

In this study, I focused on a naturally occurring population of *R. baurii* var. *confecta* to investigate whether changes in soil moisture and solar radiation (UV) facilitate changes in flower colour morphs. I addressed the following questions: 1) is there a relationship between the emergence of flower colour, soil moisture and solar radiation and 2) are there fitness differences (i.e., flower number, seed set and seed germination) between the flower colour morphs in the field and under controlled combinations of water-deficit and UV exposure conditions?

2.4 Methods and Materials

Study system

Rhodohypoxis species generally occur in mountain grasslands above 1650 m a.s.l (Hilliard & Burtt 1978). I focused on *R. baurii* (Baker) Nel., a near-endemic KwaZulu-Natal Drakensberg flowering plant species that can be distinguished by the formation of colourful carpets of white, pink and red flowers (Hilliard & Burtt 1978). These plants are perennial geophytes and die back in January to mid-October; they only produce leaves and flowers

from October-January. Although the distribution of *R. baurii* is mainly in the Drakensberg (KwaZulu-Natal) mountain grasslands located in Lesotho and South Africa, the species ranges between Barberton (Mpumalanga) and Mthata (above 1100 m a.s.l; Eastern Cape; Hilliard & Burtt 1978). *Rhodohypoxis baurii* varieties can be distinguished by their flower colour and ecological distributions in their natural populations (Hilliard & Burtt 1978). In general, flowers emerge from buds within two or three days to become fully open, and 14 days until they senesce. The inter- and intraspecific variation in flower colour among the varieties provides an opportunity to better understand the environmental variables that may contribute to the presence of FCP in this Drakensberg plant species, yet we do not know about how FCP is maintained, and why the polymorphism exists.

Here, I focused specifically on *R. baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt because individuals comprise flowers that are white, pale or bright pink (three distinct morphs), whereas *R. baurii* var. *baurii* flowers are predominantly pink and *R. baurii* var. *platypetala* flowers are predominantly white. In general, *R. baurii* individuals have more than one flowering event (multiple, independent flowers over the flowering season), and the flowers may be white, pale, or bright pink depending on the *R. baurii* variety. Originally, Hilliard and Burtt (1979) hypothesized that for *R. baurii* var. *confecta* flowers may open white and age through pink to red, however, preliminary field data suggest that individual flowers do not change colour. It appears that individual plants produce multiple flowering stems over the flowering season and these flowers are consistently the same colour morph (unpublished data). In this variety of *R. baurii*, different individuals are one of three morphs: either white, pale pink, and bright pink within a single population. Interestingly, bright pink flowers of *R. baurii* var. *confecta* are only observed in a handful of individuals. The three varieties are also characterized by their slight, but clear, distinction in both ecological habitat and geographic distribution; *R. baurii* var. *confecta* grows at high altitudes on damp, grassy

slopes that consist of partly shaded rock flushes or cliff faces, *R. baurii* var. *baurii* grows in moist, rocky habitats, whereas *R. baurii* var. *platypetala* occurs on shallow, dry, stony soils, over rock sheets.

I focused on the polymorphic population of *R. baurii* var. *confecta* in the northern Drakensberg that is located near the Sentinel Peak car park (SCP; Free State, South Africa), occurring at 2550 m a.s.l (Hilliard & Burtt 1978). My focus on a single population enabled me to control a number of variables that may have presented challenges, like habitat-related confounding factors as FCPs may be population-specific, when testing whether environmental variables facilitate changes in flower colour morphs of *R. baurii* var. *confecta* over the flowering season. At this site, *R. baurii* var. *confecta* is widespread both above and below the SCP hiking trail. The SCP population comprises thousands of *R. baurii* var. *confecta* plants with flower colour morphs ranging from white, pale pink, and bright pink. At this population, *R. baurii* var. *confecta* plants are exposed to harsh environmental conditions such as relatively low soil moisture, exposure to direct sunlight or high light conditions, and freezing temperatures even during summer months (pers. obs). Plants are exposed to direct sunlight from mid-morning to sunset (10h00-18h00) and they experience some shading in the early morning due to the surrounding mountains.

An increase in soil moisture has been shown to correspond with a shift from a majority of white flowers to a majority of pink flowers over the flowering season at this population (Gardiner & Glennon 2019). Here, in order to further test whether soil moisture and solar radiation underpin the shift in the proportion of colours in *R. baurii* var. *confecta*, I took soil moisture and solar radiation measurements in the field, to replicate in a controlled growth chamber experiment. This allowed me to examine the relationship between the relative percentages of pigmented flowers and environmental variables such as changes in soil moisture and solar radiation over the flowering season in this Drakensberg species.

In 2017, thirty mature *R. baurii* var. *confecta* plants that were at least to meters apart, were dug out and collected haphazardly from the SCP field site and repotted in a Culterra mix (50% topsoil, 50% compost); plants have been in cultivation since 2017 at the University of the Witwatersrand, Johannesburg. Plants sampled from this population were subsequently housed in a greenhouse where temperatures range between 10°C and 30°C under ambient light conditions. All plants were placed in 10 cm pots, in the same soil type. Plants in the greenhouse are watered twice a day with a sprinkler irrigation system, receiving ~ 200 ml of water twice a day (08h00, 15h30). Subsequently, the soil is maintained at a volumetric soil water content (VSWC) of ~20%; soil moisture is monitored on a weekly basis in the greenhouse. At the beginning of the flowering season (Oct), plants in the greenhouse are fertilized with a fish emulsion mixture (SeaGrow). More *R. baurii* var. *confecta* individuals were repotted and cultivated from the thirty original plants as their seeds germinated and additional plants grew in plant pots.

Flower colour change and potential environmental correlates

To establish whether the proportion of white and pink flowers changes over the flowering season, I visited the SCP *R. baurii* var. *confecta* population at three time points (early, mid, and late flowering season) between October and December in 2018 and 2019. In 2017, plants were monitored along three consecutive 50m x 1m transects, to assess flower colour through the season (Gardiner & Glennon 2019). In 2018 and 2019, I haphazardly laid out twelve one squared meter quadrats (Fig. 2.1) and, in addition, individual plants were marked to assess flower colour through the season. In each quadrat, I counted pink and white flowers (regardless of whether an individual plant comprised multiple flowers), number and colour of buds, number of senesced flowers, and number of flowers with seeds within each quadrat (Table 2.2). Where possible, I collected seeds from each quadrat into an individually marked coin envelope. Quadrats were dropped haphazardly at different positions within the

SCP population over the different visits, so it is unlikely that one individual was counted more than once. By the last trip, towards the end of the flowering season, no new flowers emerged and flowers started to senesce. I used flower number, seed set, senesced flowers and seed germination (in the greenhouse) as a measure of fitness between the white and pink colour morphs.

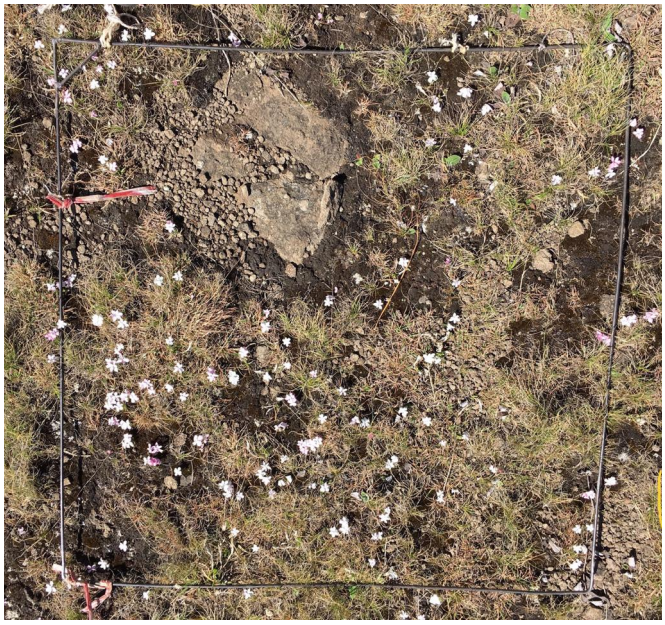

Fig. 2.1 One squared meter quadrats that were used to measure the relative counts of buds, flowers, seeds, senesced flowers of pink and white colour morphs over different visits at three time points in 2018 and 2019 at the population of *Rhodohypoxis baurii* var. *confecta* at Sentinel Peak Car Park, Free State.

Three soil moisture measures were taken haphazardly within each quadrat and the VSWC was averaged to obtain one soil moisture reading per quadrat. Soil moisture data were collected in 2017 and 2019 to compare field soil moisture and flower colour change over the flowering season. In 2018, challenges presented at the field site and soil moisture data could not be collected. Solar radiation (kJ m^{-2} d^{-1}) data were extracted from the WorldClim version 2 database (Fick & Hijmans 2017) for the SCP population. The data included three solar radiation measurements at three time points over the flowering season (Oct, Nov and Dec).

Measures of plant growth and fitness

Seed germination in the greenhouse

In 2018, mature capsules were harvested from the 12 quadrats to estimate seed production by both white and pink colour morphs. These seeds were also used to estimate germination between the colour morphs. A Culterra germination mix (50% topsoil, 50% compost) was autoclaved at 121°C for 20 minutes and stored in a sealed container in the lab. Fifty 12 cm seedling pots were filled with the germination mix and two seeds were dropped directly in pots for germination (n = 50 seeds per morph). Seedling pots were labelled and housed in a greenhouse where temperatures range between 10 °C and 30 °C under ambient light conditions, under active pest control. Seeds were watered with the irrigation system, receiving ~200 ml of water, twice a day (at 08h00 in the morning and 15h30 in the afternoon).

Growth chamber experiment

To replicate observations made at the focal field site, I conducted a growth chamber experiment where I investigated the effect of progressive water-deficit and exposure to ultraviolet (UV) light conditions, following a similar experimental design to Twyford et al. (2018). In my experiment, I used 96 *R. baurii* var. *confecta* plants that were collected from the SCP population and have been growing in the greenhouse since 2017 at the University of the Witwatersrand, Johannesburg. At the study site, average VSWC is generally 20% after it has rained and ~5% when the soil is relatively dry. As experimental drought experiments may be plant and experiment-specific, with the size and type of pot affecting soil moisture retention, among other factors, a pilot drought study on potted *R. baurii* var. *confecta* plants showed that at a VSWC lower than 10%, plants started to wilt and became photosynthetically constrained whereas plants were physiologically active (response to gas exchange measurements) at a VSWC above 10%. Subsequently, a VSWC of 10% was used as a threshold for the water-deficit treatment.

I selected two different soil moisture levels (well-watered and water-deficit) and exposure to UV light (proxy for solar radiation) to test the effect of each variable on plant growth and emergence of flower colour. Four experimental treatments were developed (Fig. 2.2). In the first treatment, plants were well watered and exposed to UV light to test for the effect of UV exposure and adequate soil moisture conditions on plant growth (UV+/W+). In the second treatment, plants were exposed to a combination of water-deficit conditions and UV exposure to enable a decoupling of both variables from each other (UV+/W-). For a third treatment, plants were well watered with 200 ml of water per day as a control for the experiment (UV-/W+). In the fourth treatment, plants experienced a water-deficit with no UV treatment to test the effect of water deficiency on plant growth (UV-/W-).

Six mature *R. baurii* var. *confecta* plants were randomly assigned to each treatment in each of the four growth chambers (n = 24 individuals per growth chamber; n = 96 plants used in the experiment). I selected and used mature plants to remove potentially confounding factors due to developmental effects. All four experimental treatments were replicated across four growth chambers. At the time of collection in the field in 2017, plants had white/very link pink flowers but did not have flowers prior to the experiment in 2019, so I was unable to confidently assign known morphs to specific experimental treatments. As such, plant maturity was based on the number of leaves (more than three leaves) and size of leaves (leaf-length of up to 2 cm).

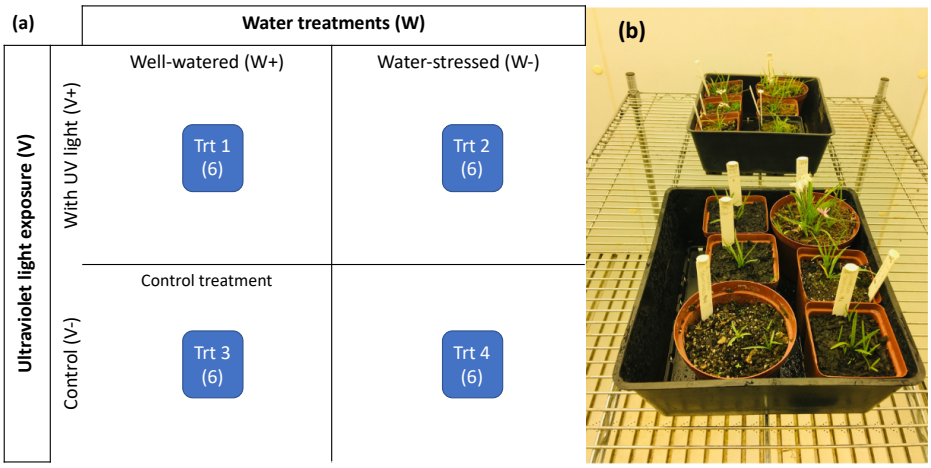

Fig. 2.2 a) Experimental design that combines two potential environmental variables, soil moisture and UV exposure, divided into four experimental treatments with six individuals per treatment. b) A layout of twelve *Rhodohypoxis baurii* var. *confecta* individuals in two water treatments in the growth chambers.

A Conviron (CONVIRON; model no PGW40) program was used to run the growth chamber experiment. Reference carbon dioxide (400 ppm) and relative humidity (60%) were maintained at controlled set-points in the program, while temperature (°C) and light intensity (incandescent and fluorescent light levels) were ramped up to simulate daily changes in a natural environment. Prior to the experiment, I downloaded Conviron general trend data, and placed iButton data loggers (Fairbridge Technologies) to record temperature in each growth chamber. This enabled me to assess if set-points and actual points on temperature and light intensity were comparable between growth chambers. A week prior to the start of the experiment, plants were moved from the greenhouse and placed into the growth chambers to allow them to acclimate to set chamber conditions. Over the course of the experiment, growth chambers and plants were regularly inspected for pests.

The experiment started in the second week of November 2019, after ensuring that Conviron general trend data and iButton temperature data matched across the growth chambers. Plants had not set flowers prior to the experiment. The experiment ran for a total of six weeks over the flowering season (between Nov–Dec 2019). The duration of the growth chamber experiment correlated with the natural flowering season of *R. baurii* var. *confecta* in the field. During the experiment, plants were randomly rotated between the four chambers, within their same experimental treatment, at weekly intervals to minimise chamber/block effect.

Normal incandescent (42w/230v) and fluorescent lights (54w/840) were used in the growth chambers, with an addition of blacklight blue, fluorescent lights (36w/1200 nm) in the

UV exposure treatments (Fig. 2.3). Plants in the UV treatments were given supplemental UV light (UV = ~ 120 nm) while plants in the control treatment were assigned to regular white lights (UV = ~ 40 nm). A digital UV light meter (UV-340A, Lutron Electronic Enterprise Co., LTD) was used to quantify UV light emitted by the normal incandescent and fluorescent lights, blacklight blue, fluorescent lights onto the plants (UV lights), and the combination of both normal and UV lights.

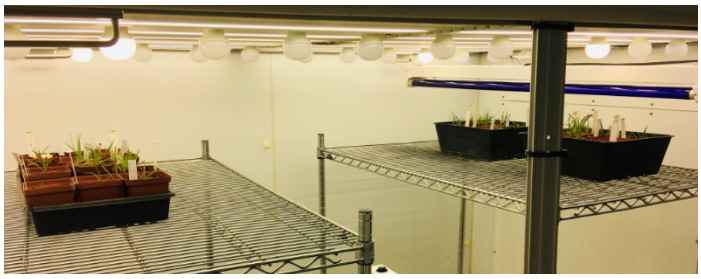

Fig. 2.3 Growth chamber experiment set up that shows a combination of ultraviolet light exposure and water conditions of 24 *Rhodohypoxis baurii* var *confecta* individuals. The experimental design was replicated across four growth chambers.

In the water-deficit conditions, plants were only watered every four days, while plants in the well-watered treatment were watered daily and received ~200 ml of water per day. Soil moisture was measured once every four days in the morning (before 09h00) for all plants across all treatments using a Hydrosense II meter with a 12 cm moisture probe (Campbell Scientific Inc., Utah). Three soil moisture measurements were taken within each pot, and the VSWC were averaged to obtain one soil moisture reading per pot.

In order to assess fitness and growth of the plants under each of the treatments, five vegetative traits were scored for each plant once per week over the six-week growth chamber experiment: number of leaves, length and width of the largest leaf, and the number and colour of floral buds/flowers.

Statistical analyses

All statistical analyses were performed in RStudio (RStudio Team 2018). All plots were made using *ggplot* (Wickham 2016) and associated packages in RStudio (RStudio Team 2018). A multiple regression analysis was used to test the relationship between the two explanatory environmental variables, soil moisture and solar radiation, and the response variable, the percentage of pigmented flowers at three time points (early, mid and late flowering season) over the flowering season. The response variable, percentage of pigmented flowers, was log-transformed to improve normality (log, natural logarithm).

Values of the traits scored/measured during and at the end of the growth chamber experiment (number of leaves, length and width of the largest leaf, and the number and colour of floral buds/flowers) were analysed separately using the Kruskal-Wallis test followed by the Kruskal-Wallis Multiple Comparison Post hoc test when significant differences were obtained. Values of the traits were further analysed as response variables using the linear mixed-effects model (lmer) function from the package lme4 (Bates et al. 2015). To account for within plant and chamber variance, and the potential temporal effect during the growth chamber experiment (measurements were taken on the same plants at different times), plant ID, week number and chamber ID were set as random effects in the models, with the four experimental treatments as a fixed effect.

Measurements of largest-leaf length were multiplied by the corresponding leaf width to estimate an approximate measurement of leaf area. Flower colour was scored as binary (0/1) data – a score of '1' assigned to pigmented (pale and bright pink) morphs and a score of '0' assigned to unpigmented (white) morphs.

2.5 Results

Flower colour change over the flowering season

Flower counts in the field

I recorded 77,000 counts of flowers at the SCP population across different visits at the three time points (October, November and December) over three years (2017, 2018 and 2019), over the entire flowering season (Oct - Dec). Of the 77,000, 47,000 pink and 30,000 white morphs were counted over the time period. When excluding the transect data collected in 2017, approximately 1600 white morphs and 3700 pink morphs were counted from the quadrat data at the SCP population in 2018 and 2019 (Table 2.2).

Data from the field showed that measures of the two explanatory environmental variables, soil moisture and solar radiation, differed between the three time points over the flowering season (Table 2.2). In general, there was a relatively higher average soil moisture and higher average solar radiation towards the end of the flowering season (Dec).

Although trends show a clear shift in the relative percentages of flowers over time, towards more pigmented (pale and bright pink) flowers in the late flowering season across the three years (Fig. 2.4), there was no significant difference in the relative percentages of pigmented flowers over the three time points ($\chi^2 = 104$, df = 100, $P = 0.37$; Fig. 2.4). However, when excluding data from the midpoint (peak) of the flowering season (Nov), there was a significant difference in the percentages of pigmented flowers between the beginning and end of the flowering season ($\chi^2 = 20.189$, df = 1, $P < 0.05$). I recorded 305 pink and 433 white flowers at the beginning of the flowering season (Oct) and 1915 pink and 724 white flowers towards the end of the flowering season (Dec; Table 2.2).

In 2018, I counted 338 seeds from white flowers and 454 seeds from pink flowers towards the end of the flowering season (Table 2.2). No seeds were observed on individuals or collected in the field during the 2019 flowering season. Although more pink flowers were counted in the field over the flowering season, pink morphs had a lower average number of seeds per individual plant (n = 0.51) than white morphs (n = 0.63; Table 2.2).

Table 2.2 Relative counts of floral buds, flowers, seeds, average number of seeds per individual plant, and senesced flowers of pigmented (P; pale and bright pink) and white (W) colour morphs measured in 12 one squared meter quadrats over different visits at three time points in 2018 and 2019 at the population of *Rhodohypoxis baurii* var. *confecta* at Sentinel Peak Car Park, Free State. Measurements of solar radiation and soil moisture were taken at three different time points over the flowering season. Soil moisture measurements were taken on one squared meter quadrats in 2019 - 2018 soil moisture data are unavailable.

Flowering season	Year	Buds		Flowers		Seeds		Average seeds/plant		Senesced flowers	Mean % Soil Moisture	Mean Solar Radiation ($kJ\ m^{-2}\ d^{-1}$)
		W	P	W	P	W	P	W	P			
Early	2018	18	213	269	196	0	0	–	–	0	–	21567
Early	2019	32	19	164	109	0	0	–	–	0	9.54	21567
Mid	2018	0	0	218	1065	0	0	–	–	410	–	23970
Mid	2019	17	12	244	348	0	0	–	–	166	14.98	23970
Late	2018	0	0	536	894	338	454	0.63	0.51	786	–	24695
Late	2019	41	123	188	1021	0	0	–	–	605	26.08	24695

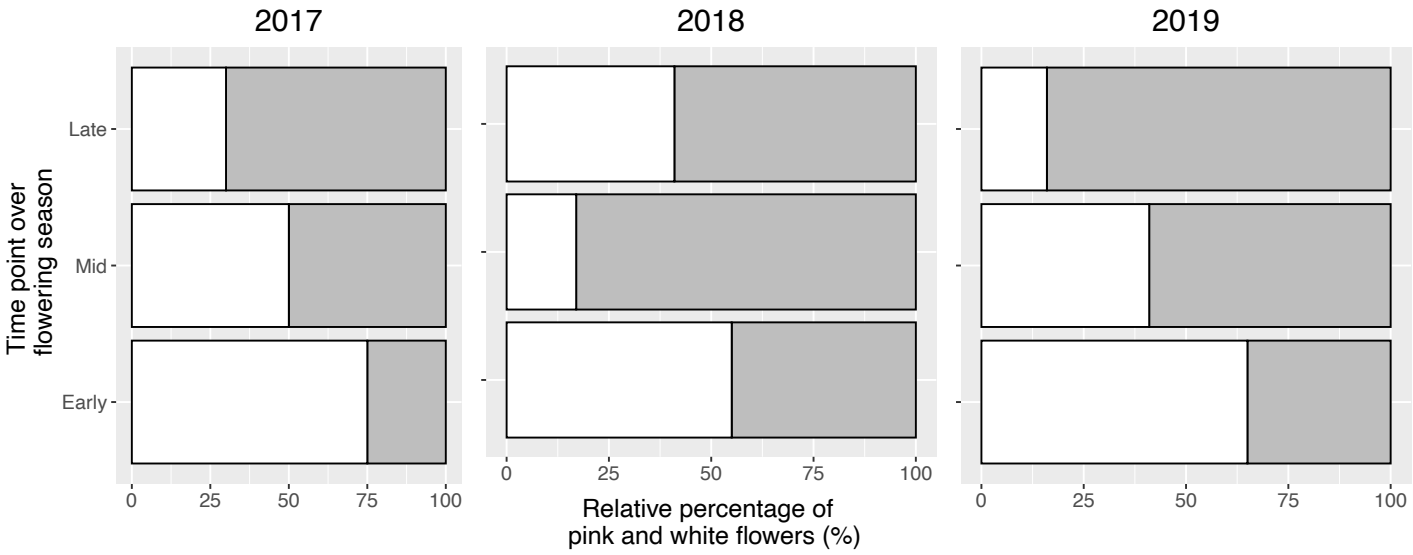

Fig. 2.4 Relative percentages of pink and white colour morphs measured over different visits at three time points in 2017, 2018 and 2019 at the population of *Rhodohypoxis baurii* var. *confecta* at Sentinel Peak Car Park, Free State. Bars represent the relative percentages of white morphs (white bars) and pink morphs (grey bars).

Flower colour change and potential environmental correlates

There was a positive relationship between mean soil moisture and the percentage of pigmented flowers in 2017 ($r^2 = 0.78$, $P = 0.0001$; Fig. 2.5) and 2019 ($r^2 = 0.12$, $P = 0.03$; Fig. 2.5). However, general comparisons showed no significant differences in mean soil moisture over the three time points ($\chi^2 = 78$, df = 77, $P = 0.45$). Soil moisture data were not available for 2018; as such, I was unable to examine the relationship between soil moisture and flower colour data over all three years.

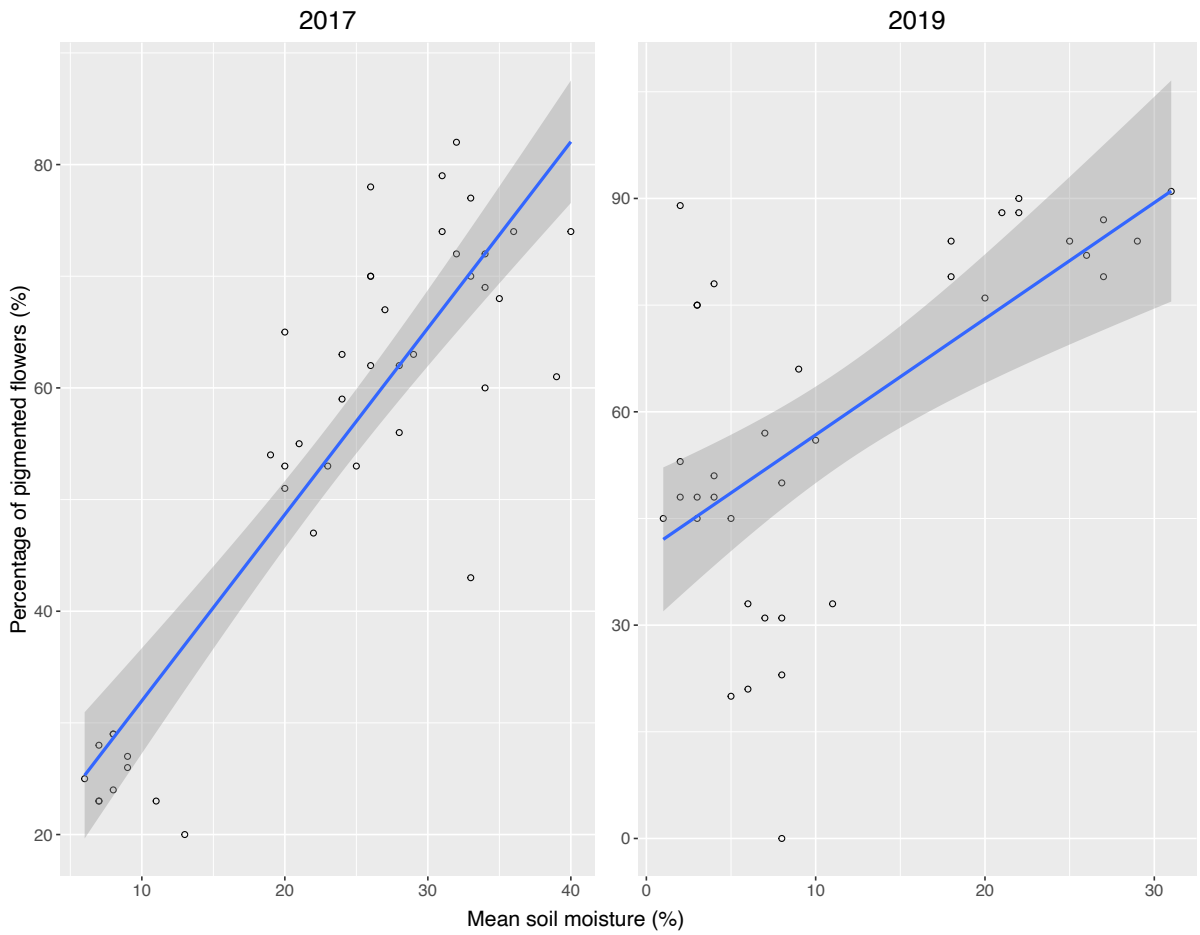

Fig. 2.5 Relationship between mean soil moisture and percentage of pigmented flowers at the population of *Rhodohypoxis baurii* var. *confecta* at Sentinel Peak car park, Free State, using the 2017 transect and 2019 quadrat soil moisture data.

Although there was a positive relationship between mean soil moisture and the percentage of pigmented flowers (Fig. 2.5), solar radiation best explained change in the relative percentages of flowers (Table 2.3). There was a significant difference in solar radiation between the three time points over the flowering season ($\chi^2 = 67.54$, df = 35, $P = 0.001$; Table 2.2).

Table 2.3 Summary of a multiple regression model on the relationship between mean soil moisture and solar radiation as predictor variables and percentage of pigmented flowers as a response variable at the population of *Rhodohypoxis baurii* var. *confecta* at Sentinel Peak car park, Free State, using the 2017 transect and 2019 quadrat soil moisture data. Asterisks denote statistically significant results at $P = 0.05$.

Source	Estimate	*SE*	*t* value	*P*
(Intercept)	-2.48e+00	2.91e+00	-0.85	0.40
Mean soil moisture	-5.65e-02	2.78e-01	-0.20	0.84
Solar radiation	2.68e-04	1.24e-04	2.17	0.03*
Mean soil moisture x solar radiation	2.57e-06	1.15e-05	0.22	0.82

Measures of plant growth and fitness

Growth chamber experiment

Mean soil moisture and UV measures differed between the four experimental treatments in the growth chambers (Table 2.4). Average VSWC was 17.49% for well-watered treatments and 3.45% for water-deficit treatments (Table 2.4). General trends showed that individuals in the well-watered treatments experienced a decline in VSWC of no less than 10% per day, whereas individuals had their VSWC decline to 0% in water-deficit treatments.

Table 2.4 Measured values of mean soil moisture and UV readings in a growth chamber experiment under four experimental treatments (UV-/W+ = well-watered conditions with no UV, UV+/W+ = well-watered conditions and UV exposure, UV-/W- = water-deficit conditions with no UV, and UV+/W- = water-deficit conditions and UV exposure). Measurements were taken at weekly intervals. Ninety-six individuals were measured over six weeks.

Treatment	Mean % Soil moisture	Mean UV (nm)
UV-/W+	16.19	40
UV+/W+	18.78	120
UV-/W-	2.70	40
UV+/W-	4.19	120

Vegetative traits measurably differed between the four experimental treatments (Table 2.5; Fig. 2.6). Individuals in the water-deficit and UV exposure treatment had a greater average number of leaves and largest-leaf area than individuals in the other three treatments (UV+/W-; Fig. 2.6). However, differences in final number of leaves and largest-leaf area may be explained by initial number of leaves (χ^2 = 15.72, df = 3, P = 0.00l) and initial largest-leaf area (χ^2 = 11.58, df = 3, P = 0.001).

Of the 96 plants used in this experiment, 72 individuals produced flowers while the other 24 individuals did not produce flowers over the course of the experiment. Overall, twenty pigmented and 113 white individual flowers (n = 133) were counted over the course of the growth chamber experiment. Flowers were roughly spread out between individuals and across the experimental treatments; 24 individuals did not produce flowers, while the other 72 plants produced between one to eight flowers over the course of the experiment. Initial flower number did not contribute to final number of flowers across treatments (χ^2 = 7.10, df = 3, P = 0.07).

Overall, individuals in the two UV treatments (UV+/W+ and UV+/W–) produced 102 flowers, of which 15 were pink, while individuals in the treatments with no UV (UV–/W+ and UV–/W+) produced 31 flowers, of which five were pink. Of the 20 pigmented flowers counted over the course of the experiment, 15 was produced by individuals in the UV treatments, the remainder (n = 5) were in the non-UV treatments. Only the well-watered and UV exposure (UV+/W+) treatment had a significant overall effect on the percentage of pigmented flowers (Table 2.5; Table S1).

No seeds were observed on individuals or collected during the growth chamber experiment.

Table 2.5 Summary of linear mixed-effects (lmer) models of the vegetative and floral responses of *Rhodohypoxis baurii* var. *confecta* to controlled combinations of water-deficit and UV exposures in a growth chamber experiment under four experimental treatments (UV-/W+ = well-watered conditions with no UV, UV+/W+ = well-watered conditions and UV exposure, UV-/W- = water-deficit conditions with no UV, and UV+/W- = water-deficit conditions and UV exposure). Plant ID, week number, and chamber ID were random effects. Standard error (*SE*) and estimate of regression coefficients (Estimate) are reported. Asterisks denote statistically significant effects of experimental treatments on the measured traits at $P = 0$ '***', $P = 0.001$'**' or $P = 0.01$'*'. Significant *P*-value means the treatment has a measurable effect on the response variable. The relevant response variable is listed above each table.

(a) Total number of leaves

Treatment	Estimate	*SE*	*t* value	*P*
UV-/W+	16.31	3.17	5.15	0 ***
UV+/W+	21.16	3.17	6.68	0 ***
UV-/W-	11.31	3.17	3.57	0 ***
UV+/W-	23.73	3.17	7.49	0 ***

(b) Largest-leaf area

Treatment	Estimate	*SE*	*t* value	*P*
UV-/W+	111.36	14.11	7.90	0 ***
UV+/W+	148.08	14.11	10.50	0 ***
UV-/W-	117.92	14.11	8.36	0 ***
UV+/W-	162.17	14.11	11.50	0 ***

(c) Total number of white and pigmented flowers

Treatment	Estimate	*SE*	*t* value	*P*
UV-/W+	0.13	0.13	1.02	0.31
UV+/W+	0.38	0.13	2.90	0.005 **
UV-/W-	0.08	0.13	0.64	0.52
UV+/W-	0.33	0.13	2.57	0.01 *

(d) Percentage of pigmented flowers (%)

Treatment	Estimate	*SE*	*t* value	*P*
UV-/W+	1.39	1.76	0.79	0.44
UV+/W+	5.90	1.76	3.35	0.002 **
UV-/W-	1.39	1.76	0.79	0.44
UV+/W-	3.13	1.76	1.77	0.08

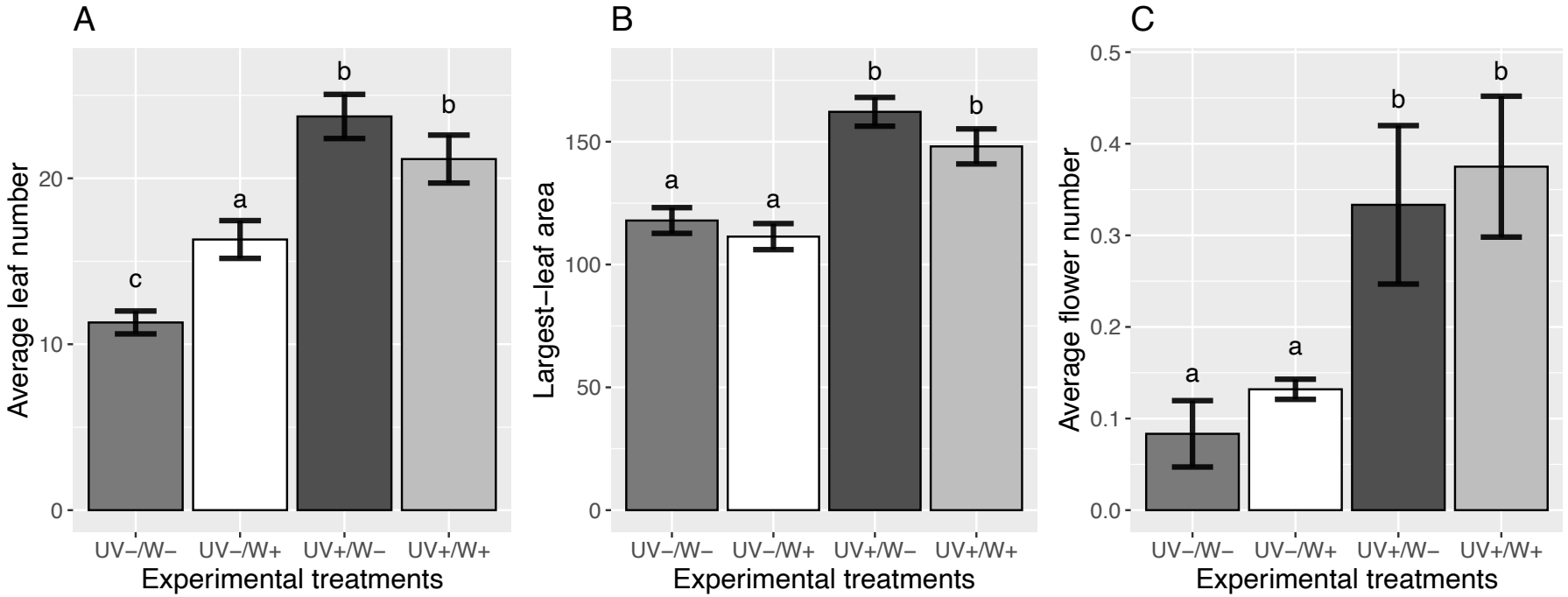

Fig. 2.6 Vegetative and floral responses of *Rhodohypoxis baurii* var. *confecta* to controlled combinations of water deficit and UV exposure in a growth chamber experiment, under four experimental treatments (UV-/W+ = well-watered conditions with no UV, UV+/W+ = well-watered conditions and UV exposure, UV-/W- = water-deficit conditions with no UV, and UV+/W- = water-deficit conditions and UV exposure). Measured traits comprised average number of leaves (A), largest-leaf area (B) and average number of flowers (C). Ninety-six individuals were measured over six weeks. Different letters indicate significantly different effects in multiple comparisons of all treatment combinations in post hoc tests ($P = 0.05$).

Individuals in the two UV treatments (UV+/W+ and UV+/W–) produced 54 (10 pink) and 48 flowers (five pink), respectively, while individuals in the treatments with no UV (UV–/W+ and UV–/W+) produced 19 (two pink) and 12 flowers (three pink), respectively. Of the 20 pigmented flowers counted over the course of the experiment, 10 were produced by individuals in the well-watered and UV exposure (UV+/W+) treatment. Although individuals in the UV and non-UV treatments produced different total numbers of flowers, the treatments produced a similar percentage of pink flowers from their total numbers of flowers produced: 31 flowers were produced in non-UV treatments (16% pink) and 102 were produced in UV treatments (15% pink).

Seed germination in the greenhouse

Fifteen seeds, or 30% of 50 seeds, from pink morph individuals and four seeds, or 8% of 50 seeds, from white morph individuals germinated after approximately eight weeks, from seeds that were collected during the 2018 flowering season. Pink morphs had a higher average number of germinating seeds per plant (n = 2.54) than white morphs (n = 0.75).

2.6 Discussion

Here, using a growth chamber set up, I attempted to replicate field conditions where soil moisture and solar radiation differ at three time points over the flowering season to further investigate whether soil moisture or solar radiation, or their interactive effect facilitated changes in flower colour morphs. In my experiment, I used UV exposure as a proxy for increased solar radiation. Combinations of water-deficit and UV exposure conditions were divided into four controlled experimental treatments to replicate environmental variables that would be similar to what the plants would experience at the focal field site at the beginning and end of the flowering season.

My findings showed that individuals in the treatments with no UV produced fewer flowers that were mostly white, while individuals in the treatments with UV exposure produced the highest total number of white and pigmented flowers. These findings are comparable with observations from the focal field site when there were more pigmented flowers when both soil moisture and solar radiation were relatively higher towards the end of the flowering season than in the early and mid-time points (Table 2.2). There was a significant difference in the percentage of pigmented flowers between the beginning of the flowering season and end of the flowering season ($\chi^2 = 20.189$, df = 1, $P < 0.05$). Although there is a correlation between increased soil moisture and increased percentage of pigmented flowers over time, soil moisture alone did not best explain the observed shift from a majority of white flowers to a majority of pink flowers ($P = 0.84$), and neither did the interaction between soil moisture and solar radiation ($P = 0.82$). As the shift in flower colour morphs may be not be linear over the flowering season, excluding the mid-season time point showed that there was a significant difference in the percentage of pigmented flowers between earlier in the flowering season and later in the season.

In my experiment, while the UV treatments had a significant effect on the total number of white and pigmented (regardless of morph) flowers that were produced, only the well-watered and UV exposure treatment (UV+/W+) had a significant overall effect on the percentage of pigmented flowers (Table 2.5). These findings are what I expected because towards the end of the flowering season at the SCP population, there are generally more pink flowers after it had rained, and the sun angle corresponded to high light conditions and more UV exposure. Previous work by Gardiner & Glennon (2019) on *R. baurii* var. *confecta* indicated that solar radiation and other environmental variables such as temperature and Julian date did not primarily contribute to the observed changes in flower colour in the field, and that soil moisture better explained the shift in flower colour morphs over the flowering

season. Here, I found that solar radiation best explained the shift in flower colour morphs in *R. baurii* var. *confecta*.

Solar radiation induces the production of anthocyanins, the most common floral pigments in plants (Chalker-Scott 1999). Previous work suggests that anthocyanin production prevents UVB damage (reviewed in Chalker-Scott 1999). For example, plants may accumulate anthocyanins in the leaf epidermis to prevent damage from UV radiation (e.g., Takahashi & Badger 2011). Anthocyanins may also contribute to UV protection by absorbing parts of UVA and UVB spectra, thereby providing an effective sunblock (UV-screening; Takahashi & Badger 2011). Consequently, it may be advantageous to produce pigmented colour morphs in stressful environmental conditions such as relatively high light conditions or increased solar radiation. Further, my findings showed that plants in the UV exposure treatments produced more and larger vegetative traits (i.e., higher number of leaves and largest-leaf area) than plants in the non-UV treatments, indicating that plants modify their traits in response to environmental variation, to increase survival and fitness (e.g., Picotte et al. 2007). In some instances, both drought and UV exposure can facilitate plant growth (e.g., increased stolon production in a Monkey flower population; Twyford et al. 2018).

The ability to regulate the expression of anthocyanins may enhance plant response to harsh environmental conditions, potentially because anthocyanin production may be costly in environments that are not stressful (Warren & Mackenzie 2001). Consequently, one would expect pigmented flowers in stressful environments, like increased solar radiation, as they are better able to tolerate heat and water-deficit environmental conditions (Warren & Mackenzie 2001; Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Rausher 2008). For example, in the two colour morphs (white and blue) of *Linanthus parryae*, blue-flowered plants had higher fitness than white-flowered plants in poor years (i.e., years of low spring precipitation – blue morphs produced more seeds than white morphs (Schemske &

Bierzychudek 2001). A conventional pattern was observed in *R. baurii* var. *confecta*, with the proportion of pink flowers increasing under increased solar radiation conditions. One would expect to find the pink flowers of *R. baurii* var. *confecta* in drier environments (i.e., reduced soil moisture), however, soil moisture also increased as the flowering season progressed. As *R. baurii* var. *confecta* plants are geophytes and occur on grassy rock slopes at high altitudes above 2600 m a.s.l in mountain grasslands (Hilliard & Burtt 1978), increased solar radiation and higher soil moisture (that may induce water clog, bulb rot), but not water stress are probably the most critical to their chance at survival and reproduction over the flowering season. Although I did not explore other environmental variables, in addition to those investigated in previous work by Gardiner & Glennon (2019) in the field, it is unlikely that factors such as soil pH and soil type would differ enough to facilitate changes in flower colour morphs at a microhabitat scale over the flowering season, within a single locality. The extremely close nature of the white and pink morphs at the SCP population, located within 2 cm of each other, suggests that soil pH is unlikely to change significantly over the flowering season or within my sampled quadrats.

Linking fitness measures to environmental stressors may enable us to better predict the outcomes of plant responses to different environmental variables, especially in non-model plant systems like the Drakensberg *R. baurii* var *confecta*, as flower colour differences are frequently associated with plant fitness (Strauss & Whittall 2006; Arista et al. 2013). Further, we may better predict the consequences of plant responses to different environmental pressures. One such consequence is the difference in fitness levels between white and pigmented flower colour morphs (e.g., Schemske & Bierzychudek 2001; Strauss & Whittall 2006), although the effects (direct or indirect) of FCP on fitness can be complicated (see Forsman 2016).

Seeds from both of the colour morphs germinated under greenhouse conditions. However, more seeds from pink flowers germinated relative to seeds from white flowers (30% vs. 8%), which could be attributable to the conditions in the greenhouse; seeds likely got more water than they would have in the field, which accelerated germination. As pink flowers are more likely under higher solar radiation/UV conditions in the field, it is possible that conditions in the greenhouse, at the time of my seed germination experiment, may have been comparable to the mid- and early flowering season in the field (high light conditions and more UV exposure), allowing for seeds from pink morphs to germinate. However, future work to germinate seeds under different light/UV conditions would be helpful to test this hypothesis. On average, pink flowers produced more seeds than white flowers in the field, under increased solar radiation over the flowering season (mid-late December; Table 2.2) but had a lower average number of seeds per individual plant compared to white morphs. However, pink morphs had a higher average number of germinating seeds per plant, which might relate, directly, to plant fitness towards the end of the flowering season. This means that although white morphs may produce more seeds towards the end of the flowering season in the field, most of the seeds have a lower germinating rate compared to seeds produced by pink morphs. No seeds were observed on individuals or collected in the field during the 2019 flowering season, likely because the flowering season might have shifted later in the year than expected due to low rainfall.

The SCP population comprises predominately white flowers at the start of the flowering season and predominantly pink flowers as the flowering season progresses, which could translate into population persistence in response to potentially harsh environmental conditions in nature through morphs that have a differential tolerance to environmental change. The presence of FCP examined here occurs within populations of a single species and is present throughout the lifetime of a plant. I cannot exclude the possibility that FCP in

this Drakensberg species is due to the presence of pollinators; some pollinators might preferentially visit one colour morph over the other. However, preliminary observations have not revealed any obvious pollinators at the focal field site that may be pollinating *R. baurii* var. *confecta,* and artificial pollination does not induce flower colour change either (pers. obs). The floral morphology of these plants, coupled with the lack of nectar reward, suggests that pollinators are not necessary for seed production and that flowers are unlikely pollinated at night. Further, preliminary data in the lab showed that *R. baurii* var. *confecta* is self-compatible, yet seeds are produced from crosses between white and pink colour morphs. In addition, I did not observe any signs of herbivory on the leaves and flowers in the field. These findings provide an opportunity to study FCP and how it relates to environmental variables in the absence of biotic factors, like pollinators.

I emphasize that my focus on a single population allowed me to remove potential confounding effects from different populations. However, it is possible that my results may be population-specific and an outcome of adaptation to environmental conditions encountered at the study site. Therefore, by transplanting the plants of both the white and pink morphs in the greenhouse under controlled environmental conditions (e.g., humidity, temperature), I aimed to remove potentially factors related to environmental variation and pre-adaptation. Under controlled combinations of water-deficit and UV exposure, there was a non-random occurrence of white and pink morphs across treatments, without knowing the plants' initial colour. However, while a total of 102 flowers were produced by plants in the UV exposure treatments, only 31 flowers were pink. Interestingly, while individuals in the UV and non-UV treatments produced different numbers of flowers, they each produced a similar percentage (16% and 15% for non-UV treatments and UV treatments, respectively) of pink flowers of their total numbers of flowers produced. One would expect that all morphs in the UV

treatments would be pigmented and a higher percentage of pigmented flowers to be found in UV treatments.

The plants I used did not have flowers prior to the experiment, and I would not have been able to assign known morphs to specific treatments. Also, plants that were collected in the field had flowers that were primarily white or very light pink. As such, my assortment of plants in the experiment, without knowing initial plant's flower colour morph, may have influenced the outcomes of the experiment. This might also explain why there were no bright pink flowers emerging in the experiment. Although pink flowers are more abundant at the end of the flowering season in natural populations, white morphs are also observed, which might explain why there were white morphs in the UV+ treatments. Also, it is possible that UV exposure may not always lead to pigmentation in flowers. Nevertheless, these findings support preliminary data that indicated that individual flowers do not change colour, and that individuals produce flowers of the same morph over the flowering season. In my experiment, a single plant consistently grew the same flower colour morph throughout the experiment.

Solar radiation was the best predictor variable of the percentage of pigmented flowers in the field, while the well-watered and UV exposure treatment (UV+/W+) had a significant effect on the percentage of pigmented flowers that were produced during the experiment. My findings suggest that as anthocyanin production may be costly in environments that are not stressful (Warren & Mackenzie 2001), pink flowers in *R. baurii* var. *confecta* were more likely under higher solar radiation/UV and high soil moisture conditions in the field and growth chamber experiment. According to these findings, it is possible that pink morphs may be more capable of surviving stressful environments (e.g., increased solar radiation) than unpigmented morphs. FCP may be an adaptation for these plants to take advantage of increased solar radiation/UV towards the end of the flowering season, in order to increase seed production. Flower pigmentation in the early flowering season when UV levels are

relatively lower than in the mid- and late time points of flowering season would be wasting anthocyanins. Future work to repeat the growth chamber experiment using known colour morphs is necessary to further disentangle the effect of solar radiation and soil moisture on flowers colour morphs in this study system.

2.7 Conclusion

This study showed that pigmented (pink) colour morphs of *R. baurii* var. *confecta* are more prevalent towards the end of the flowering season and that increased solar radiation in the field and UV exposure, under controlled combinations of water-deficit and UV exposure in a growth chamber experiment, facilitate the shift in flower colour morphs over time. When *R. baurii* var. *confecta* individuals were acclimatized to similar environmental factors (e.g., light intensity, humidity, temperature) in a greenhouse to remove potentially confounding environmental factors and then exposed to controlled combinations of water-deficit and UV exposure in the growth chambers, I found that UV exposure influenced plant growth and the production of pigmented flowers was more prevalent in UV exposure treatments than in non-UV treatments. *Rhodohypoxis baurii* plants are long-lived and probably manage stress through stored resources, thus, future work to characterize how to define stress for these plants will provide further insight into changes in environmental variables and their complex interplay in inducing a stress response in this study system. Nevertheless, my research offers insights into the relationship between FCPs and environmental variables and how these relationships maintain FCP in a polymorphic mountain endemic plant population, although the relationship may be population-specific.

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CHAPTER THREE

Assessing locus-specific population differentiation in a mountain endemic plant

Sentinel Peak in the Free State province, South Africa, where a polymorphic population of *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt occurs (Photo: MP. Mtileni)

3.1 Abstract

Identification of outlier loci that may be responsible for genetic variations is useful to gaining an understanding of evolutionary and ecological processes driving trait polymorphisms. Here, I performed an amplified fragment length polymorphism (AFLP) scan in three naturally occurring populations of the polymorphic near-endemic Drakensberg plant species, *Rhodohypoxis baurii* var. *confecta* with different extents of flower colour polymorphisms (FCPs; one polymorphic population and two monomorphic populations), to search for loci that may demonstrate atypical among-population genetic differentiation and may be potentially influenced by selection ('outlier loci'). Of the 113 loci scored from three different primer combinations, only one outlier locus was detected in the *R. baurii* var. *confecta* genome scan with no statistical significance. Interestingly, the outlier locus was found in 80% and 30% of individuals from the flat catchment and Witsieshoek lodge populations (the monomorphic populations), respectively, while only 8% of individuals in the polymorphic population possessed the outlier locus. Further, no differences among populations were significant, with lower molecular variance among populations (3%). These findings illustrate that FCPs in *R. baurii* var. *confecta* are likely population-specific and may not be attributable to locus-specific population differences.

3.2 Keywords

AFLP, environmental variables, flower colour polymorphism, Hypoxidaceae, mountain grasslands endemic, population genetics

3.3 Introduction

As plants may adapt to environmental change through trait variation (e.g., Picotte et al. 2007), like flower colour polymorphism (FCP hereafter) within a population (Schiestl & Johnson 2013), one would expect genome-wide allele frequency differences at loci between populations that occur in different environments (Storz 2010). Feng et al. (2015) suggest that these shifts in allele frequencies across genomes and between populations can be identified using population genetic approaches. Therefore, investigating FCP and potential environmental correlates across heterogeneous environments would first require identifying anonymous loci that have a unique pattern of genetic differentiation that may suggest that these are under selection (termed 'outlier loci'; e.g., Storz 2005; Feng et al. 2015). The genetic underpinning of FCP (reviewed in Wennersten & Forsman 2012) may form the basic population variation necessary for selection to take place, as morphs may have an increased adaptive potential capacity (Forsman et al. 2008). As such, there may be a positive relationship between potentially adaptive traits, like FCPs, and plant persistence and adaptation in the face of environmental change, outside of pollinator preference (Forsman 2016) and variation in anonymous loci that may be potential outlier loci (Feng et al. 2015).

An accumulation of a variety of pigments (e.g., betalains, carotenoids and anthocyanins) determine flower colour (Grotewold 2006), with anthocyanins being the most visible and conserved among angiosperms (Holton & Cornish 1995; Winkel-Shirley 2002). For example, the Mediterranean Basin comprises 87.7% of plant species that have anthocyanin based FCPs (Narbona et al. 2018). Anthocyanins are produced by the anthocyanin biosynthesis pathway (ABP; Grotewold 2006; Rausher 2008), where a combination of at least eight specific genes work together to produce anthocyanins (Dooner & Robbins 1991; Grotewold 2006) - most of the bright red, pink and blue colours (Dooner & Robbins 1991; Holton & Cornish 1995). Anthocyanins may be compartmentalized in various

tissues of the plant, primarily leaves, flowers and seeds (Chalker-Scott 1999) and may form the basis of FCP, where two or more discrete variants of flower colour (termed 'morphs' hereafter per Narbona et al. 2018) may coexist in at least one population (Narbona et al. 2018). In some instances, the frequency of morphs in a single species may vary through time (e.g., Schemske & Bierzychudek 2001). Ford (1945) and Huxley (1955) suggest that morphs are unlikely due to recurring mutations.

A near-endemic Drakensberg plant genus, *Rhodohypoxis* Nel. (Hypoxidaceae), an important component of the Drakensberg mountain grasslands genera (Hilliard & Burtt 1978), is a useful system to assess genetic differentiation patterns between populations with different extents of FCPs. This small genus comprises six species, among which *R. baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt shows striking FCP (white, pale and bright pink morphs over the flowering season; Hilliard & Burtt 1978). *Rhodohypoxis* is potentially threatened as the mountain grasslands it occurs in may be threatened by global change (for an example see O'Connor 2005).

Amplified fragment length polymorphisms (AFLPs) are a robust, quick, transferrable and cost-effective molecular technique for screening of genetic diversity widely distributed across genomes (Vos et al. 1995; Poncet et al. 2010; Blignaut et al. 2013), to obtain allele frequency data and generate potential outlier loci (Meudt & Clarke 2007) even with little or no knowledge of the organism's genome (Vuylsteke et al. 2007; Schlötterer 2004; Poncet et al. 2010). AFLPs can be applied to almost all organisms across species' ranges (Bensch & Akesson 2005; Meudt & Clarke 2007; Vuylsteke et al. 2007), with the capacity to generate large amounts of genome-wide allele frequency data (Mueller et al. 1999) with potential outlier loci across the genome (Feng et al. 2015). Here, I compared AFLP-based genetic diversity among three populations of *R. baurii* var. *confecta* where one population experiences a marked shift in white and pink morphs (a shift from a majority of white flowers

to a majority of pink flowers over the flowering season) and two monomorphic populations do not experience a prominent shift in colour (flowers remain predominantly white over the flowering season; Gardiner & Glennon 2019). The goal of this study was to search for loci that are divergent among the three populations that vary in FCP. I addressed whether there are locus-specific population genetic differences between populations that may exist in a population where a change in pink and white colour morphs occurs versus those populations that do not experience a colour change. Specifically, I assessed the AFLP data to identify potential outlier loci among the three populations.

3.4 Methods and Materials

Study system

Rhodohypoxis Nel. (Hypoxidaceae) species are herbaceous perennial geophytes that exist primarily throughout the Drakensberg mountain grasslands located in Lesotho and South Africa (Hilliard & Burtt 1978). Six *Rhodohypoxi*s species make up this small genus (Hilliard & Burtt 1978). Here, I focused on the potentially most widespread South African near-endemic species, *R. baurii* (Baker) Nel. Hilliard & Burtt, whose distribution ranges between the Mpumalanga and Eastern Cape provinces of South Africa, with the KwaZulu-Natal Drakensberg as its main centre of distribution. *Rhodohypoxis baurii* comprises three varieties, among which *R. baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt is the only variety that shows considerable and identifiable FCPs (i.e., sheets of mixed white, pale and bright pink flowers over the flowering season; Hilliard & Burtt 1978). This variety grows on high altitude (2250 m a.s.l.) damp, grassy slopes that consist of partly shaded rock flushes or cliff faces, and leaves are erect and bright green (leaf length and width average 25–75 mm and 1.5–5 mm, respectively; Hilliard & Burtt 1978). Flowers may open white and age through pink to red (Hilliard & Burtt 1978), however, preliminary data suggest this is not the case (unpublished data). In general, flowers emerge from buds within two or three days to

become fully open and after approximately 14 days, they senesce. For this study, I focused on three populations of *R. baurii* var. *confecta* that occur at a single locality (Sentinel Peak/Witsieshoek area) in the Free State province, South Africa (Hilliard & Burtt 1978); this locality accommodates thousands of *R. baurii* var. *confecta* plants. The three populations occur within 4.62 km of each other (Gardiner & Glennon 2019).

The first population is located near the Sentinel Peak car park (SCP; Free State), in the northern Drakensberg. At this site, thousands of *R. baurii* var. *confecta* plants are widespread both above and below the SCP hiking trail at 2550 m a.s.l and are exposed to direct sunlight from mid-morning to sunset (10h00–18h00), as the surrounding mountain cliffs provide some shading in the early morning (Gardiner & Glennon 2019). The other two populations, one located on a flat catchment at 2249 m a.s.l and the other behind Witsieshoek lodge at 2195 m a.s.l, occur at relatively lower altitudes compared to the SCP population, and are only exposed to direct sunlight until mid-afternoon when the sun is blocked by the escarpment. These two populations do not experience a marked shift in white and pink morphs (flowers remain predominantly white) compared to the SCP population which experiences a marked shift in white and pink morphs over the flowering season (Oct–Jan; Gardiner & Glennon 2019). Solar radiation/UV exposure may explain the shift from a majority of white flowers to a majority of pink flowers over the flowering season (see Chapter Two results).

Environmental variables

To compare environmental variables among the three *R. baurii* var. *confecta* populations at the Free State locality, environmental data were required for all sampling locations, per population, at different time points (early, mid- and late flowering season) over the flowering season (Oct-Dec). I used data from Gardiner & Glennon (2019) where geographical coordinates (latitude and longitude), temperature and soil moisture

measurements were recorded during different visits at each population over the 2017 flowering season. Briefly, one 50 m x 1 m transect was haphazardly laid out at each population. Soil moisture measurements were taken using a Hydrosense II meter with a 12 cm moisture probe (Campbell Scientific Inc., Utah) at every 10 m along a 50 m x 1 m transect per population, to obtain a volumetric soil water content (VSWC) reading. Overall, five VSWC measurements were taken per transect, from which mean soil moisture, minimum and maximum soil moisture were calculated per population. Temperature was recorded by placing an iButton data logger (Maxim iButton DS1923, Fairbridge Technologies) at each population. Towards the end of the flowering season (Dec), temperature data were downloaded using OneWireViewer version 1.5 (Maxim Integrated), from which minimum and maximum temperature, mean temperature were calculated per population. Solar radiation (kJ m^{-2} d^{-1}) data were extracted from the WorldClim version 2 database (Fick & Hijmans 2017), resulting in three measurements (Oct, Nov and Dec) per population.

Plant material collection & DNA extraction

In 2017, leaves were collected haphazardly from 10–30 mature *R. baurii* var. *confecta* individuals (two-three leaves per individuals, per population) into individual bags with silica desiccant. At each population, leaves were collected from whole plants that occurred at least two meters from one another. All leaf material was desiccated on silica desiccant gel and stored in the lab, at room temperature, until DNA extractions took place. In 2020, 25 individuals from the polymorphic SCP population, 10 from the lodge and 10 from the catchment populations (n = 45), where tissue was enough, were prepared for DNA extractions. DNA was extracted from leaf material using the standard CTAB extraction protocol per Doyle & Doyle (1987) and Cullings (1992). I prepared CTAB buffer, weighed out 10–20 mg of silica-dried leaf tissue and ground the tissue with blue pestles, aiming to

grind the tissue into powder - grinding was done with the aid of liquid nitrogen (see Appendix I for specific details).

AFLP analysis

AFLPs were generated following Blignaut et al. (2013). The protocol comprises three steps: 1) using restriction enzymes to digest the genome, followed by ligation of adapters to restricted fragments, 2) pre-selective DNA amplification of the restricted fragments and 3) DNA amplification using primers with three additional nucleotides to selectively amplify fragments (see Appendix II for specific details). All samples were sent to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa, for visual measurement of selective amplification products to detect fragments (loci) and their sizes using a 3500xl DNA Sequencer and the ROX size standard.

Fragment sizing & scoring

Fragment data from CAF were analysed using Geneious Prime 2020.2.4 (Biomatters, Auckland, New Zealand). Fragments (loci) of sizes between 50–400 bp were scored manually to ensure the presence or absence of a band. In the subsequent data matrix, a score of '0' represents an absence of a fragment and a score of '1' represents the presence of a fragment. This resulted in a binary (0/1) dataset for 113 loci from all 45 individuals from three populations.

Outlier detection

Computer programs, like BayeScan (Foll & Gaggiotti) and DetSel (Vitalis et al. 2003), have been developed to detect markers responding to selection, and to identify outlier loci from genome-wide scans using dominant makers, like AFLPs. Specifically, BayeScan, a Bayesian and reversible-jump Monte Carlo Markov chain (RJMCMC) model which is based on the multinomial-Dirichlet distribution, has the capacity to detect a usually high percentage

of outlier loci from allele frequency data and has been evaluated and reported to be relatively efficient compared to the other methods (Pérez-Figueroa et al. 2010). Here, I used BayeScan (version 2.1; Foll 2012) to detect potential outlier loci among the three *R. baurii* var. *confecta* populations, to address whether there are among-population genetic differences. The following parameters were set in the BayeScan model: number of pilot runs (-nbp 20), the number of outputted iterations (-n 5000), thinning interval size (-thin 10), length of pilot runs (-pilot 7000), burn-in length (-burn 90000) and prior odds for the neutral model (-pr odds 10). Outputs from the model were text files including the posterior probability odds (log10 (PO) for the model, locus-specific component shared by all populations (α), false discovery rate (FDR) analogue of the p-value (q-value) and the level of differentiation among/between populations (F_{ST}) to estimate directly the probability that each locus is under selection (Foll 2012). Convergence tests on my binary matrix data showed that the MCMC converged, indicating that the data were solid to produce conclusive outputs.

Genetic diversity & genetic differentiation

GenAlEx (version 6.5; Peakall & Smouse 2006, 2012) was used to estimate measures of genetic diversity and genetic differentiation - the expected (He) heterozygosity across all loci for each population, level of differentiation between/among populations using Nei's (1972) genetic distance matrix (D) and percentage of polymorphic loci (%) for each population and generate a principal coordinate analysis (PCoA) to assess whether populations clustered separately. An analysis of molecular variance (AMOVA) was used to calculate the proportion of genetic variance within and among populations using ΦPT values (analogues to Wright's F_{ST} when data are binary; Excoffier et al. 1992; Peakall & Smouse 2006).

3.5 Results

Environmental variables

Data from the field showed differences in environmental variables measured at the three populations over the flowering season (Table 3.1). The SCP population showed the lowest average minimum temperature (1.37 °C) and the highest average maximum temperature (36.28 °C) compared to the flat catchment population and Witsieshoek lodge population (3.32 – 35.28 °C and 3.91 – 30.57 °C, respectively). Solar radiation was highest at the flat catchment population compared to the other two populations (Table 3.1).

Table 3.1 Sampling site (population), altitude, geographic coordinates, average minimum., maximum and mean temperature, average min., maximum and mean soil moisture, solar radiation and sample size per population. Soil moisture measurements were taken at every 10 m along a 50 m x 1 m transect, yielding five volumetric soil water content (VSWC) measurements per sampling site.

No.	Sampling site	Alt. (m a.s.l)	Lat.	Longitude	Temp (°C)			Moisture %			Solar	N
					Min	Max	Mean	Min	Max	Mean	Radiation ($kJ\ m^{-2}\ d^{-1}$)	
1	Sentinel Peak	2550	28°43’39”S	28°53’29”E	1.37	36.28	18.82	17.20	30.14	23.67	24695	25
2	Witsieshoek lodge	2195	28°41’11”S	28°53’57”E	3.91	30.57	17.24	12.14	20.83	16.48	24970	10
3	Flat catchment	2249	28°42’14”S	28°53’37”E	3.32	35.28	19.30	10.22	17.77	14.01	25510	10

Average minimum soil moisture was lowest (10.22%) at the flat catchment population and highest (30.14%) at the SCP population. Overall, the SCP population had the highest mean soil moisture (23.67%) while the flat catchment population had the highest average maximum temperature over the flowering season (19.30 °C; Table 3.1).

Outlier detection

A total of 113 fragments (loci) were scored from the three primer combinations for the 45 individuals sampled, of which the mean number of the 113 loci was 152.88. Percentage of polymorphic loci was 91.15%, 85.84% and 93.81% for the flat catchment, Witsieshoek lodge and Sentinel peak populations, respectively (Table 3.2). Overall, I obtained a total of 102 polymorphic loci.

Table 3.2 Sampling site (population), sample size per population and percentage of polymorphic loci for three *Rhodohypoxis baurii* var. *confecta* populations at 113 loci.

No.	Sampling site	N	Polymorphic loci (%)
1	Sentinel Peak	25	93.81
2	Witsieshoek lodge	10	85.84
3	Flat catchment	10	91.15

Only one outlier locus, the 94th locus (133), was detected and identified in the BayeScan program among the three populations (Fig. 3.1, 3.2), however, there were no outlier loci detected within each population (Fig. 3.2). The outlier locus was found in a total of 13 (28%) of the 45 individuals sampled, of which eight (62%) were from the flat catchment population, three (23%) were from the Witsieshoek lodge population and two (15%) were from the Sentinel peak population. Overall, 80% of individuals in the flat catchment population possessed the outlier locus, while only 30% and 8% of individuals from the Witsieshoek lodge population and Sentinel peak population, respectively, possessed the locus.

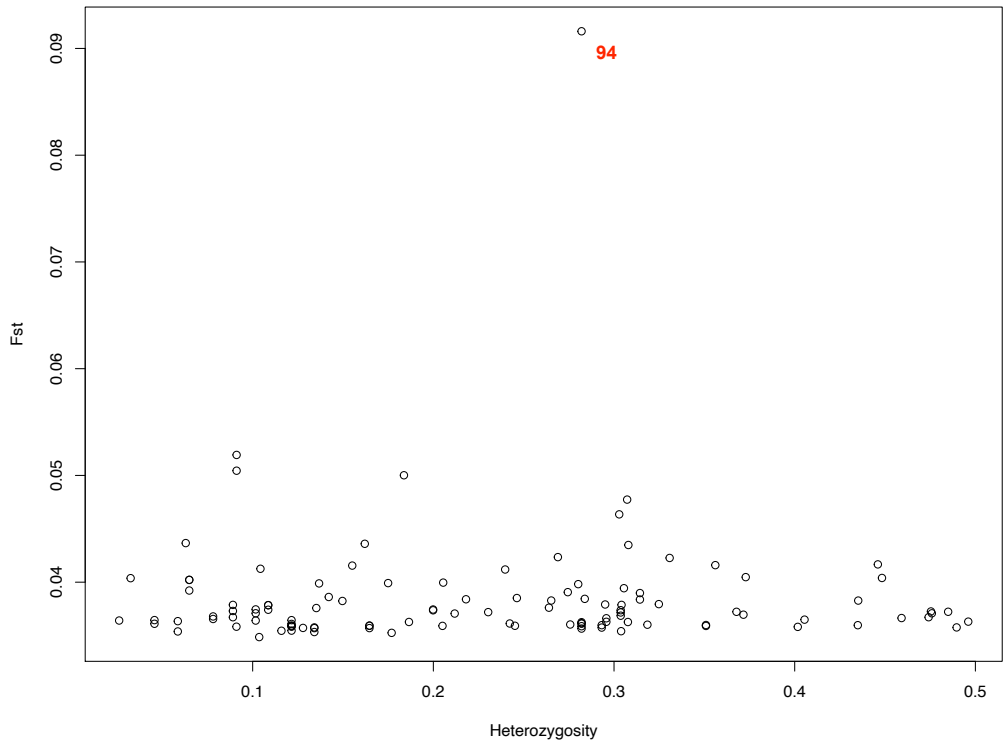

Fig. 3.1 Distribution of F_{ST} values as a function of expected heterozygosity for the three *Rhodohypoxis baurii* var. *confecta* populations at 113 loci. Only one locus, the 94th locus (locus 133) was identified as an outlier potentially under selection.

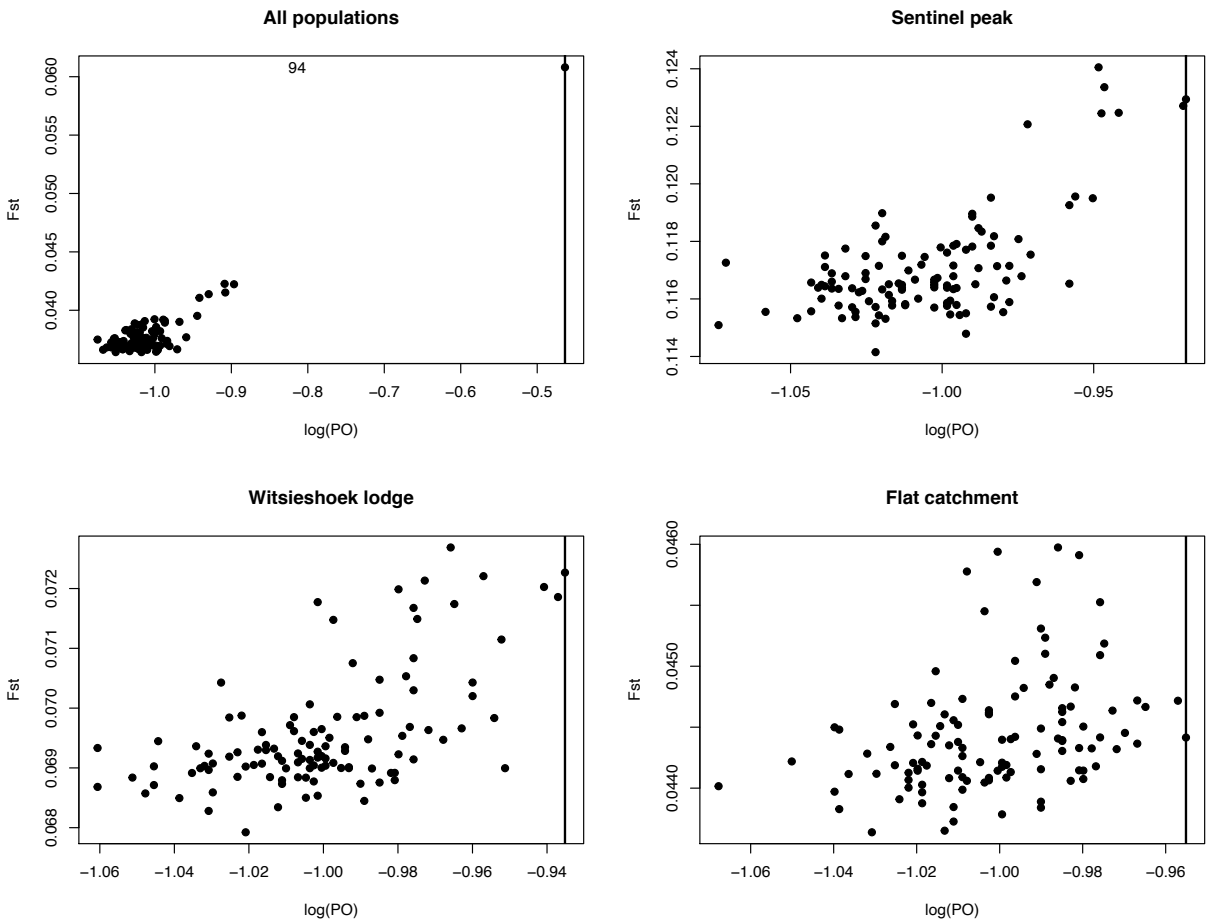

Fig. 3.2 BayeScan plots of the amplified fragment length polymorphism data of 45 individuals from three *Rhodohypoxis baurii* var. *confecta* populations – Sentinel peak, Witsieshoek lodge and flat catchment. F_{ST} is plotted against the log10 of the posterior odds (PO) for all three populations and then separately for each population.

BayeScan outputs for the outlier locus showed the highest alpha value ($\alpha = 0.758$; locus-specific component shared by all populations), highest level of differentiation (F_{ST} = 0.091), highest posterior probability ($\log_{10}$ (PO) = 0.144) and the lowest q-value (= 0.418) compared to the other loci (Table 3.3, S2).

Table 3.3 Locus-specific component shared by all populations (α), level of differentiation (F_{ST}), q-value and posterior probability (log10 (PO) for loci under natural selection for three *Rhodohypoxis baurii* var. *confecta* populations.

No.	Locus	q-value	α	F_{ST}	Log10 (PO)
94	133	0.418	0.758	0.091	0.144

Genetic diversity & genetic differentiation

Mean He values were 0.265, 0.217 and 0.203 across all loci for the flat catchment, Witsieshoek lodge and Sentinel peak populations, respectively – it was highest at the flat catchment population. Genetic distance value between the Sentinel peak population and Witsieshoek lodge population was lowest (D = 0.009), indicating that the two populations are genetically similar while genetic distances between the flat catchment population and Sentinel peak population, flat catchment population and Witsieshoek lodge population were highest and equal (D = 0.036 and 0.035, respectively; Table 3.4).

Table 3.4 Pairwise population matrix of Nei's (1972) genetic distance (D) for three *Rhodohypoxis baurii* var. *confecta* populations at 113 loci.

Flat catchment	Witsieshoek lodge	Sentinel peak	
-			Flat catchment
0.035	-		Witsieshoek lodge
0.036	0.009	-	Sentinel peak

Results of the AMOVA (Table 3.5) indicated that most (97%) of the molecular variation in *R. baurii* var. *confecta* exists within populations, with lower molecular variance

among populations (3%). The overall ΦPT was not significant (ΦPT = 0.028, $P > 0.001$; Table 3.5), which indicates that no differences among populations are more than expected by random. Similar results were observed on the principal coordinates analysis (PCoA) based on genetic distance, which indicated that individuals from the three populations formed two clusters - one where the three populations cluster together and one with the Sentinel peak and Witsieshoek lodge populations, although individuals from the flat catchment population appear to be further away from individuals of the Sentinel peak and Witsieshoek lodge populations (Table 3.4; Fig. 3.3).

Table 3.5 Analysis of molecular variance (AMOVA) for three *Rhodohypoxis baurii* var. *confecta* populations at 113 loci ($P = 0.001$). (df =degree of freedom, SS = sum of squares, MS = mean squares, Est. Var. = estimate of variance, % variation = percentage of total variation among populations based on 999 permutations, and ΦPT = proportion of the total variance between populations).

Source	df	SS	MS	Est. Var.	% variation
Among populations	2	47.802	23.901	0.503	3%
Within populations	42	722.420	17.200	17.200	97%
Total	44	770.222		17.703	100%
ΦPT = 0.028					
P (rand > data)					

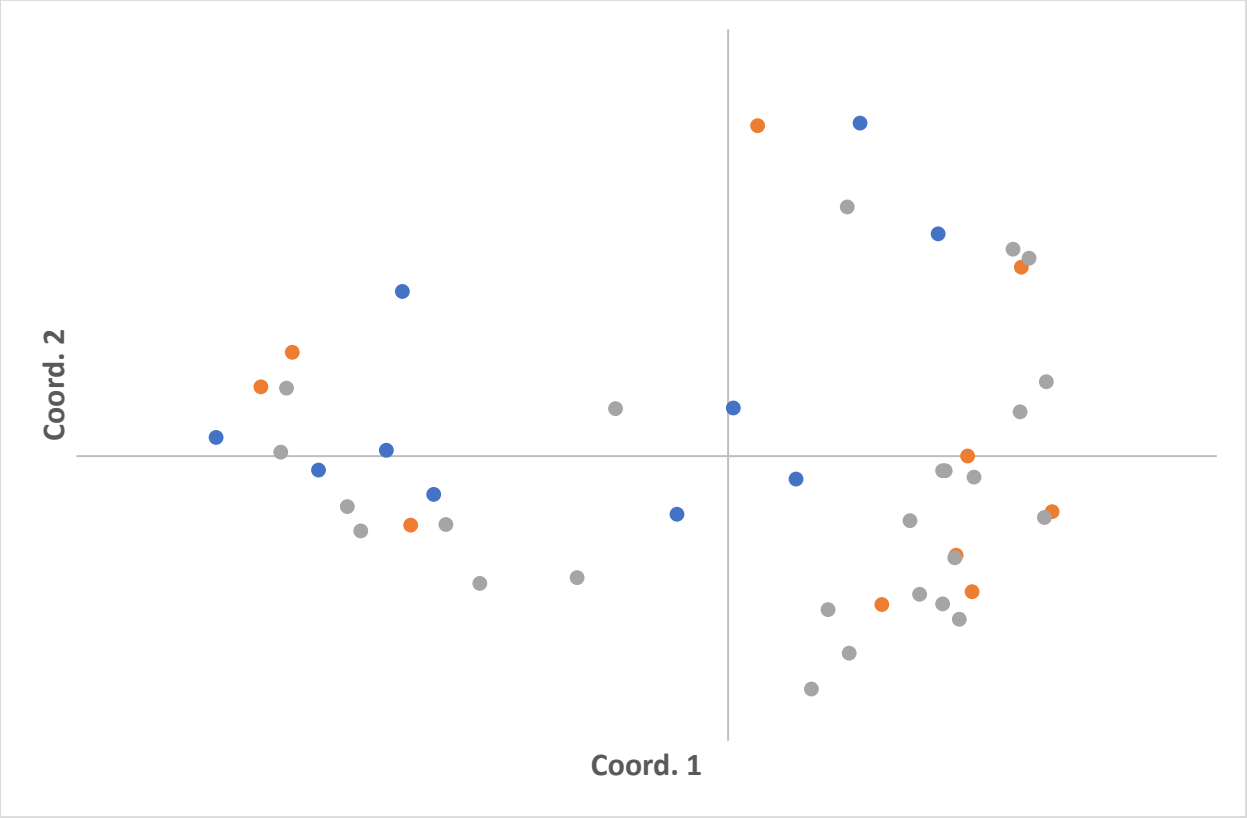

Fig. 3.3 Population structure of 45 individuals from three *Rhodohypoxis baurii* var. *confecta* populations at a single locality in the Free State province, South Africa - Sentinel peak (grey), flat catchment (blue), and Witsieshoek lodge (orange): Principal coordinate analysis (PCoA) at 113 loci.

3.6 Discussion

In response to environmental change, through the process of evolution, plants may adapt by modifying their morphological and physiological traits (e.g., Picotte et al. 2007). As such, plant traits, like FCP (Schiestl & Johnson 2013) can be viewed as a marker for a plant responding to environmental change, while environmental variables may be potential selection agents. As plants may respond and subsequently adapt to different environmental conditions (specific to their natural environments), one would expect genetic differences among populations found in different environments (Storz 2010). Specifically, populations may contain genome-wide allele frequency differences at loci that can be identified using population genetics analyses, like those implemented in BayeScan (Feng et al. 2015). These molecular methods have the capacity to generate large amounts of allele frequency data across genomes using codominant or dominant markers, like AFLPs, with the ability to detect and identify outlier loci that may be under natural selection (Feng et al. 2015).

I compared AFLP-based genetic diversity (e.g., Feng et al. 2015, Zhang et al. 2018) of three *R. baurii* var. *confecta* populations that occur at a single locality in the Free State province of South Africa, yet they show different extents of FCPs. Of the three populations, only the SCP population experiences a marked shift in white and pink morphs over the flowering season (Oct–Dec) whereas the flat catchment and Witsieshoek lodge populations do not– colour morphs are predominantly white over the flowering season. Findings from the field indicated that there are interpopulation differences in environmental variables measured at the population sites over the flowering season, and that increased solar radiation may explain the shift in flower colour morphs over the flowering season (see Chapter Two results). Here, mean temperature was highest at the flat catchment population and lowest at the Witsieshoek population while mean soil moisture was highest at the SCP population and lowest at the flat catchment population (Table 3.1). Overall, all three populations experience

hot (maximum temperatures > 30 °C) and wet (maximum soil moisture > 15%) over the flowering season, although the SCP occurs at the highest altitude (2550 m a.s.l) and appears to be the wettest and hottest (Table 3.1) than the other two populations. Solar radiation was highest at the flat catchment population (Table 3.1). One would expect the SCP population to show outlier loci that can be associated with changes in flower colour morphs and associated environmental variables. As such, investigating population differentiation using outlier loci is an important step to address alternate explanations of potential population differences in natural populations (Zhang et al. 2018).

Although there was lower molecular variance and insignificant genetic differences among the three populations (Table 3.5), mean expected heterozygosity was highest (He = 0.265) at the flat catchment population, and the PCoA based on genetic distance indicated that individuals from the flat catchment population are more different from individuals of the other two populations (Fig. 3.3). Specifically, genetic distance values were highest and equal between the flat catchment and Witsieshoek populations, and between the flat catchment and SCP populations (D = 0.035 and 0.036, respectively) and lowest between the Witsieshoek lodge and SCP populations (D = 0.009, Table 3.4) indicating that the two populations are genetically similar (Nei 1972).

Of the 113 loci scored from three different primer combinations, only one outlier locus was identified among the three populations, with no outliers within each population (Fig. 3.1, 3.2). Interestingly, the outlier locus appears to be found in the majority of individuals from one of the two monomorphic populations - the flat catchment population, as eight out of 10 of individuals sampled in this population possessed the outlier locus. BayeScan outputs for the outlier locus indicated this locus is potentially an outlier when compared to the other loci across the three populations (Table 3.3; Table S1). Although the positive value of alpha suggests diversifying selection (Foll 2012), a log10 (PO) that is less

than 0.5 suggests that the selection is negligible (Foll 2012) while the low F_{ST} coefficient of 0.091 (possibly due to insufficient data or not enough populations) makes it difficult to detect balancing selection from genome scan outliers (Foll & Gaggiotti 2008). The outlier locus also showed the lowest q-value (analogues to the p-value) compared to the other loci, however, none of the q-values across loci (including the outlier) were significant at a threshold of 10%. Together, these findings indicate that although the flat catchment population does not appear to be genetically similar to the Witsieshoek lodge and SCP populations, there are no significant genetic differences and molecular variation among the three populations. Further, my findings also indicate that the outlier locus found at the flat catchment population may not be influenced by selection. To my knowledge, the flat catchment population is flatter and so it gets more solar gain, and it is heavily grazed. The constant sun exposure also means soil is drier than if on a rocky slope like the SCP population. This may explain the potential genetic divergence from the other two sites. However, future research is necessary to investigate geological and environmental variables at localized scales that may be related to the outlier locus. Nevertheless, as the three populations are so close together and the rest of the AFLPs suggest high gene flow among them, the outlier locus is unlikely to be adaptive.

Increasing the number of individuals per population and including additional monomorphic and polymorphic populations may be useful in providing sufficient data that may be representative of each population and associated environmental variables in the field, to find potential outlier loci from AFLP scans. Further, using a sufficient number of different primer combinations and comparing simulations of different computer programs and associated models, their assumptions and biases, to assess the influence of statistical significance level on the outcomes of AFLP-based genomic scans (e.g., Herrera & Bazaga 2008) may also provide informative sets of outliers.

As I do not necessarily know what the potential outlier loci may be associated to in this study system, it is necessary to further investigate if there are outlier loci that may be under selection, and to determine their function and potential involvement in the local adaptation of *R. baurii* var. *confecta*. Further, investigating the potential effects of environmental variables on the molecular mechanisms of the outlier loci would be useful to ascertain if the outliers may be population-specific, and related to environmental variables or traits other than FCP. Compiling a comprehensive database on phenotypic, ecological and genomic data in monomorphic and polymorphic populations of *R. baurii* var. *confecta* requires further data collection.

Nevertheless, my findings indicated that FCP at the SCP population may not be attributable to locus-specific population differences as the three populations (two monomorphic populations and one polymorphic population) are genetically similar, and even under strong divergent selection, the outlier locus is likely related to morphological and physiological traits other than FCP as morphs at the flat catchment and Witsieshoek populations, but not the SCP population are predominantly white over the flowering season.

3.7 Conclusion

My findings showed that although the three *R. baurii* var. *confecta* populations show different extents of FCPs, there are no locus-specific population differences among them. However, the Witsieshoek lodge and SCP populations appear to be more genetically similar to one another than they are to the flat catchment population - there were no significant genetic differences and molecular variation among the three populations Overall, my research highlights that FCP at the SCP population is not currently linked to selection on anonymous AFLP loci, and that the presence of outlier loci does not necessarily translate into divergent selection in populations or species. Nevertheless, my research offers insights into the ecological and genetic relationship in a mountain grassland endemic.

3.8 References

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CHAPTER FOUR

General Discussion and Conclusions

4.1 Overview & general discussion

It is becoming increasingly necessary to examine the ecological and evolutionary explanations of FCPs across species ranges, especially since the diversity of floral traits, FCPs and subsequent floral evolution continue to form the basis of interesting and important discoveries in evolutionary biology (e.g., Wright 1943; Kay 1978; Schemske & Bierzychudek 2001; Strauss & Whittal 2006; Rausher 2008; McKinnon & Pierotti 2010; Sobel & Streisfeld 2013; Carlson & Holsinger 2015; Ng & Smith 2018). Interestingly, species may be monomorphic in some populations and polymorphic in others (Wright 1978; McLean & Stuart-Fox 2014; Forsman 2016) or comprise varying frequencies of different flower colour morphs in populations through time (e.g., Schemske & Bierzychudek 2001). In general, for a species to be considered polymorphic, two or more morphs should coexist in at least one population (Narbona et al. 2018). Previous work on FCPs has mainly focused on model systems or plant species in the Mediterranean Basin (e.g., Narbona et al. 2018), with a few studies on the non-model plant systems (e.g., Schemske & Bierzychudek 2001; Hopkins & Rausher 2011; Hopkins et al. 2012). Here, I focused on a Drakensberg near-endemic plant species, *R. baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt, to study how ecological factors lead to evolutionary consequences of FCPs (e.g., Strauss & Whittall 2006), including reproductive isolation (Lowry et al. 2018), selection (Servedio et al. 2011) and subsequent floral evolution (Schemske & Bradshaw 1999; Fenster et al. 2004; Schiestl & Johnson 2013). This study aimed to contribute to a growing body of work that investigates the relationship

between FCPs and environmental variables (e.g., Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Narbona et al. 2018) and compares fitness between morphs (Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Arista et al. 2013).

Briefly, I investigated whether changes in solar radiation and soil moisture, outside of pollinator preference, may explain the shift from a majority of white flowers to a majority of pink flowers over the flowering season in a polymorphic population (Chapter Two), and compared AFLP-based genetic diversity among three discrete populations (Chapter Three) of *R. baurii* var. *confecta*. Preliminary observations at the study site have not identified pollinators that may be visiting *R. baurii* var. *confecta*, and hand-pollinations between morphs do not induce flower colour change (pers. obs). I expected that there would be no obvious pollinators that may be visiting *R. baurii* var. *confecta* as the floral morphology of these plants and the lack of nectar reward suggest that pollinators are not actively recruited and the structure of reproductive features may be indicative of selfing (pers. obs). Preliminary data in the lab indicate that *R. baurii* var. *confecta* is self-compatible. Together, these findings provide an opportunity to study FCP and how it relates to environmental variables, outside of pollinator preference.

This study had three objectives: 1) to establish whether fitness differed between white and pink flower colour morphs within the same population, 2) to establish whether exposure to solar radiation (UV) and different soil moisture levels facilitated a shift in flower colour morphs (from a majority of white flowers to a majority of pink flowers) over the flowering season (Oct-Dec) and 3) to compare genetic diversity among three populations where one population experiences a marked shift in white and pink morphs and two populations do not. I concluded that in a polymorphic population of *R. baurii* var. *confecta*, pink morphs are more fit than the white morphs and that exposure to solar radiation (UV) and different soil moisture levels may explain the shift in flower colour morphs over the flowering season.

However, FCPs in this system may not have a genetic underpinning as there was no genetic differentiation and molecular variance among the three sampled populations, although they show different extents of FCPs.

In the field, I found that pink flowers became more prevalent and produced more seeds than white flowers under increased solar radiation and soil moisture over the flowering season (mid-late December). No seeds were observed on individuals or collected in the field during the 2019 flowering season, likely because the flowering season might have shifted later in the year than expected perhaps due to limited rainfall in 2019. In the 2018 flowering season. I counted 792 seeds, of which 338 and 454 were from white and pink morphs, respectively. While, white morphs had a higher average number of seeds per individual plant than pink morphs, pink morphs had a higher average number of seeds that geminated per plant. Under controlled conditions in the greenhouse, more seeds from pink flowers germinated relative to seeds from white flowers. Specifically, 15 (30%) out of 50 seeds from pink morphs and four (8%) out of 50 seeds from white morphs individuals germinated after eight weeks in the greenhouse, under ambient light conditions. Of the 77,000 flowers counted in the field over three years (2017, 2018 and 2019), 47,000 pink and 30,000 white were counted over the time period. In my growth chamber experiment, only the well-watered and UV exposure treatment (UV+/W+) had a significant effect on the percentage of pigmented flowers. Interestingly, this treatment is similar to the late flowering season of *R. baurii* var. *confecta* when there are more pink morphs than in the early and mid-flowering season; in the late flowering season, there is an increase in solar gain as the sun moves over the season, and these flowers run on a 'north-south' mountain chain and the rainy season can often lead to an acumination of moisture in the soil.

Together, my findings indicate that there are more pink morphs than white morphs as the flowering season progresses, and that solar radiation (UV) and soil moisture could be

potential selection agents that facilitate the prevalence or maintenance of FCPs in this near-endemic Drakensberg species. My findings suggest that polymorphic populations may be responding to changes in environmental variables (e.g., the drier field season yielded more white flowers for longer). As such, it was expected that pink morphs would be more prevalent and produce more seeds than white morphs as the flowering season progresses. FCPs are associated with plant fitness, with an increased potential for pigmented morphs to be more fit than unpigmented morphs under harsh environmental conditions (Strauss & Whittal 2006; Arista et al. 2013). I found that although white morphs had a higher average number of seeds per plant, pink morphs had a higher average number of germinating seeds per plant than white morphs.

Linking FCPs and fitness measures to environmental variables may enable us to predict the consequences of plant responses to different environmental variables (potential selection agents) as flower colour differences are associated with plant fitness (e.g., Strauss & Whittal 2006; Arista et al. 2013). For example in my study, the proportion of pink morphs under higher solar radiation (UV) conditions increased towards the end of the flowering season relative to white morphs, where more seeds germinated from pink morphs than white morphs. A similar pattern was observed in the blue and orange morphs of the scarlet pimpernel, *Lysimachia arvensis,* where blue morphs are predominant in the Mediterranean (xeric environments) and orange morphs are predominant in Central Europe (wet and sunny environments) (Arista et al. 2013). In addition, in "poor" years (i.e., years of low spring precipitation), blue morphs of the dessert annual *Linanthus parryae* are more fit than white morphs (Schemske & Bierzychudek 2001). These findings suggest that pigmented morphs perform relatively better than white morphs in stressful environments.

Anthocyanins, one of three groups of compounds that determine flower colour (Grotewold 2006), may enhance stress tolerance to heat, cold and water-deficit conditions

(Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008). Specifically, anthocyanins absorb parts of UVA and UVB spectra, thereby providing UV-screening in hight light conditions (Takahashi & Badger 2011) and have been shown to be drought resistant (Chalker-Scott 1999). As such, the polymorphic SCP population may be able to cope with the environmental conditions, like solar radiation and soil moisture, as they change over the flowering season (Levin & Brack 1995; Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008; Arista et al. 2013), and may be potentially less vulnerable to increased solar radiation and high water stress compared to monomorphic populations (Forsman et al. 2008; Wennersten & Forsman 2012). Environmental variables may be key determinants of the prevalence of monomorphic and polymorphic populations in this system, as my data suggest that FCPs in *R. baurii* var. *confecta* likely enable populations to respond to changes in abiotic factors over a growing season.

If plants may exhibit trait variation, like exhibit FCPs within a population (Schiestl & Johnson 2013), to adapt to environmental change (e.g., Picotte et al. 2007), one would expect genome-wide allele frequency differences at loci between populations that occur in different environments (Storz 2010) – i.e., anonymous loci that show a unique genetic differentiation pattern and may be under selection ('outlier loci'). Here, I found that only one outlier locus was detected of 113 AFLP loci scored for three populations of *R. baurii* var. *confecta*, but it is unlikely to be influenced by selection. Interestingly, the majority, eight (62%), of the 13 individuals that possessed the outlier locus were from the flat catchment (one of the monomorphic populations) while only two (8%) of 25 individuals sampled in the polymorphic SCP population possessed the outlier locus. Therefore, if the outlier locus were under selection, it would indicate that selection could be influencing morphological and physiological traits other than FCP as the flat catchment does not experience a marked shift

in flower colour morphs over the flowering season. In a similar study, an AFLP-based genome scan of 14 discrete populations of *Viola cazorlensis* identified nine outlier loci that exhibited signatures of selection, and these loci revealed adaptive floral divergence (Herrera & Bazaga 2008).

It is possible that depending on the environmental variables that plants experience at each population, *R. baurii* var. *confecta* may have the ability to shift between phenotypes (from white to pink morphs) in response to environmental cues, like increased solar radiation, over the flowering season (Forman et al. 2008). However, while FCP may be population-specific in this system, it may be a result of plant response to environmental change and not necessarily due to a genetic underpinning. This would explain the lack of genetic diversity and differentiation across the sampled populations as selection is likely due to the FCPs resulting from genetic polymorphism (Forsman et al. 2008). However, the shift in morphs does not appear to be random as my findings from the field and growth chamber experiment show that FCP is related to changes in solar radiation and soil moisture over the flowering season. Therefore, I cannot exclude the possibility that anthocyanin accumulation and subsequent FCPs in *R. baurii* var. *confecta* may be a plastic trait (e.g., *Silene littorea*; Del Valle et al. 2018a). In polymorphic populations, *R. baurii* var. *confecta* may show varying frequencies of white and pink morphs in response to environmental change through time (e.g., *Linanthus parryae*; Schemske & Bierzychudek 2001). However, in order to draw any clear conclusions, phenotypic, ecological and genomic data need to be collected. Nevertheless, my findings suggest that variation in plasticity may not be associated with genomic differences in *R. baurii* var. *confecta*, since environmental variables (soil moisture and solar radiation) have been shown to affect the expression of FCP. Through FCP, selection should favour genotypes that contribute to the ability to increase anthocyanins and subsequent FCPs to increase fitness (e.g., viable seed production).

As the source of FCP does not affect the capacity of polymorphic populations to persist in changing environments compared to monomorphic populations (e.g., Wennersten & Forsman 2012), the white and pink morphs of *R. baurii* var. *confecta* may have a differential tolerance to abiotic conditions such as increased solar radiation. Specifically, pink morphs may be more capable of tolerating and adapting to stressful environmental conditions, hence the shift from a majority of white morphs to a majority of pink flowers as the flowering season progresses and solar radiation is highest towards the end of the flowering season (Forsman et al. 2008; Wennersten & Forsman 2012). Outside of pollinator preference, floral morphs may have a differential tolerance to environmental stressors such as drought or hot conditions (Levin & Brack 1995; Schemske & Bierzychudek 2001; Warren & Mackenzie 2001; Strauss & Whittall 2006; Rausher 2008; Arista et al. 2013). As previous work suggests that FCPs are associated with plant fitness (e.g., Schemske & Bierzychudek 2001; Strauss & Whittall 2006; Rausher 2008), it is necessary to further investigate FCP effects on the fitness of individuals, alternative colour morphs, populations and species as some morphs may be more fit than others, and potentially under selection. More research is also needed to investigate the complex interplay of other factors such as genetic drift and natural selection on flower colour divergence and floral evolution (Schemske & Bierzychudek 2001; Rausher 2008).

4.2 Concluding remarks

My research highlights that FCPs in *R. baurii* var. *confecta* may be due to population response to environmental change. Future research to investigate whether the same anthocyanin biosynthesis pathway (ABP) genes and their *trans*-acting transcription factors are expressed in white and pink colour morphs is necessary to gain an in-depth understanding of anthocyanin biosynthesis and subsequent FCPs in this system. Further, investigating and comparing environmental variables across monomorphic and polymorphic populations will

enable a deeper understanding of the significance of environmental variation in relation to FCPs. It is also necessary to investigate the potential role of genetic drift and natural selection on FCP, and the complex interplay of factors that may affect FCP maintenance in nature. My research offers insights into the ecological and genetic relationship of FCPs and how these FCPs are maintained in a mountain endemic plant.

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Appendices

Table S1. Summary of linear mixed-effects (lmer) models of the vegetative and floral responses of *Rhodohypoxis baurii* var. *confecta* to controlled combinations of water-deficit and UV exposures in a growth chamber experiment under four experimental treatments (UV-/W+ = well-watered conditions with no UV, UV+/W+ = well-watered conditions and UV exposure, UV-/W- = water-deficit conditions with no UV, and UV+/W- = water-deficit conditions and UV exposure). Plant ID, week number, and chamber ID were random effects. Standard deviation (*SD*) and variance are reported. Low variance in random effects means there was mostly no effect from random effects. The relevant response variable is listed above each table.

(a) Total number of leaves

Random effect	Variance	*SD*
Plant ID	1.604e+02	12.66
Week number	1.871e+01	4.33
Chamber ID	.591e-03	0.10

(b) Largest-leaf size

Random effect	Variance	*SD*
Plant ID	3571.59	59.76
Week number	214.79	14.66
Chamber ID	17.53	4.19

(c) Total number of white and pigmented flowers

Random effect	Variance	*SD*
Plant ID	0.31	0.56
Week number	0.01	0.11
Chamber ID	0.00	0.00

(d) Percentage of pigmented flowers (%)

Random effect	Variance	*SD*
Plant ID	0.00	0.06
Week number	0.00	0.02
Chamber ID	0.00	0.00

Appendix I: DNA extractions using the standard CTAB extraction protocol per Doyle & Doyle (1987) and Cullings (1992).

I prepared CTAB buffer, weighed out 10-20 mg of silica-dried leaf tissue and ground the tissue with blue pestles, aiming to grind the tissue into powder – grinding was done with the aid of liquid nitrogen. I then added volumes of 500 µl of the prepared CTAB buffer directly to the samples, containing PVC, CTAB and ß-merc, and ground the samples a bit more. All samples were incubated all samples at 55°C (in Eppendorf tubes) for 60 minutes, after which volumes of 500 µl of 24:1 chloroform: Iso Amyl Alcohol were added directly to the samples, then each sample was mixed well by shaking the tube, followed by a 10-minute centrifuge at maximum speed (14000 rpms). Following centrifugation, three layers could be seen from the samples - the chloroform (bottom layer), debris and proteins (middle layer) and the aqueous phase (top layer).

The aqueous phase was pipetted off into a new, labelled Eppendorf tube per sample, and the volume for each sample was estimated. Volumes of 0.08 µl cold 7.5 ammonium acetate were then added directly to each sample, followed by 0.54 µl of cold isopropanol. All samples were then mixed well by shaking tubes and stored in the freezer at -20 °C for 45 minutes, and subsequently centrifuged for three minutes at maximum speed. The next step was to pour off the isopropanol and ammonium acetate liquid, being careful not to lose the pellets with the DNA. Volumes of 700 µl of cold 70% ethanol were added to each sample, and each sample was mixed well by shaking the tube and subsequently centrifuged for one minute at maximum speed. The cold 70% ethanol liquid was poured off, being careful not lose the pellets with the DNA. The last step was to dry the pellets by inverting samples on a Kim-wipe and letting them stand until dry. Samples (pellets with the DNA) were then resuspended with 100 µl of TE buffer and allowed to resuspend at -20 °C overnight.

All resuspended DNA pellets in TE buffer were quantified using a Thermo Scientific NanoDrop^{TM} 100 Spectrophotometer (Thermo Scientific, Germany) to obtain DNA concentration (ng/µl) and purity and absorbance ($A_{260/280}$ ~ 1.8 and $A_{260/230}$ ~ 2.0), using 2 µl of each sample. The remaining volumes of samples were stored at –20 °C until AFLP analysis.

Appendix II: AFLP analysis using the protocol by Blignaut et al. (2013. Each step was visualized on a 1% agarose gel to confirm digestion, and then confirm presence of amplified fragments.

1) *Digestion & ligation*

For each DNA sample (~ 200 ng), *EcoRI* and *M*sel were used for a sequential double digestion where 1 µl of DNA was digested in a 20 µl tube, using 10 µl of a digestion mix containing 0.25 µl of *EcoRI* (Fermentas), 2 µl of *EcoRI* reaction buffer and 16.75 µl of distilled water (dH_2O), and incubated at 37 °C for 1 hour. Following incubation, a 10 µl *M*sel digestion mix containing 1 µl of *M*sel, 3 µl of Cutsmart buffer and 6 µl of dH_2O was prepared and added directly to the digestion reaction, after which all samples were incubated at 37 °C for 1 hour. Samples were further incubated at 65 °C for 30 minutes to stop enzyme activity.

A 10 µl ligation mix, containing 0.1 µl of T4 DNA ligase (Fermentas), 1 µl *M*sel adapter, 6.9 µl of dH_2O, 1 µl *EcoRI* adapter and 1 µl of T4 DNA ligase buffer, was then added to the digestion and *M*sel digestion mix. Samples were vortexed, centrifuged for 1 minute and then stored in a refrigerator at 4 °C overnight.

1) *Pre-selective DNA amplification*

A 15 µl pre-selective PCR reaction was prepared with 1X Kapa *Taq* Readymix (KapaBiotech, Cape Town, South Africa), 1 µl *M*sel + 0 primer, 1 µl *EcoRI* + 0 primer, 2.5 µl of the diluted digestion-ligation mix, and pre-selective amplification was done in sequential steps: 1) initial denaturation at 95 °C for five minutes, 2) 23 cycles of denaturation at 94 °C for 30 seconds, 3) annealing step at 56 °C for 30 seconds, 4) initial elongation at 72 °C for 30 seconds and 5) final elongation at 65 °C for 30 minutes. Volumes of 5 µl of pre-selective products were used as templates for selective amplification.

2) Selective DNA amplification

Here, half of the pre-selective amplification samples, those that produced good smears, were diluted with dH_2O (1:19). A 20 µl selective PCR reaction, containing 0.25 µl of fluorescently labelled *Eco*R1+ *M*sel selective primers - *Eco*RI-AAT (Ned™, Applied Biosystems)/ *Mse*l-CTT, *Eco*RI-ATG (6-Hex™, IDT)/ *Mse*l-CTT and *Eco*RI-CAT (Fam™, IDT)/ *Mse*l-CTT (see Blignaut et al. (2013) for all primers required for a standardized AFLP protocol), 5 µl selective PCR template, 1X Kapa *Taq* Readymix and 1 µl of unlabelled *Mse*l-CTT was prepared, and PCR reactions were done with 30 repeat cycles of PCR amplification, following PCR steps above. A separate selective PCR reaction was prepared for each selective primer.

Table S2: Locus-specific component shared by all populations (α), level of differentiation (F_{ST}), q-value and posterior probability (log10 (PO) for loci under natural selection for three *Rhodohypoxis baurii* var. *confecta* populations. A total of 113 loci were scored from three primer combinations. The outlier locus is highlighted.

Locus No.	Probability	log10(PO)	q-value	alpha	F_{ST}
1	0.238848	-0.50335	0.745658	-0.048972	0.037302
2	0.22925	-0.52661	0.7504	-0.062319	0.036304
3	0.24245	-0.49479	0.74241	-0.076352	0.035762
4	0.23445	-0.51393	0.74811	-0.006489	0.038398
5	0.23185	-0.52025	0.74981	-0.034196	0.037257
6	0.24365	-0.49196	0.74115	-0.10749	0.034856
7	0.23585	-0.51055	0.74773	-0.066255	0.036251
8	0.25305	-0.47008	0.71381	-0.033712	0.037944
9	0.24365	-0.49196	0.74115	-0.078019	0.035747
10	0.23365	-0.51587	0.74868	-0.040848	0.036859
11	0.24905	-0.47932	0.73214	0.069334	0.042269
12	0.24705	-0.48398	0.73549	-0.086713	0.035762
13	0.24285	-0.49385	0.74192	-0.065673	0.036129
14	0.24265	-0.49432	0.74217	-0.067004	0.035907
15	0.22384	-0.54	0.75205	0.029255	0.039435
16	0.25385	-0.46825	0.71174	-0.068723	0.036272
17	0.23805	-0.50526	0.74624	-0.033298	0.037418
18	0.24205	-0.49574	0.74288	0.021934	0.039914
19	0.24485	-0.48914	0.73849	-0.063831	0.036717
20	0.24545	-0.48773	0.7378	-0.068576	0.036404
21	0.24925	-0.47886	0.73038	-0.078348	0.036348
22	0.24845	-0.48072	0.73318	-0.065581	0.036442
23	0.24945	-0.4784	0.72972	-0.085005	0.035698
24	0.23265	-0.5183	0.74906	-0.037097	0.037225
25	0.24865	-0.48025	0.73267	-0.059709	0.036552
26	0.25085	-0.47515	0.72578	-0.076058	0.036408
27	0.23865	-0.50383	0.74585	-0.088685	0.035329
28	0.24425	-0.49055	0.74005	0.039087	0.040395
29	0.26185	-0.45009	0.67142	0.081237	0.043483
30	0.24745	-0.48305	0.73506	-0.035865	0.037365
31	0.23365	-0.51587	0.74868	0.007362	0.039065
32	0.24145	-0.49716	0.74377	-0.040772	0.037061
33	0.22985	-0.52514	0.75001	-0.07498	0.035654
34	0.24425	-0.49055	0.74005	-0.015814	0.038281
35	0.24645	-0.48538	0.73669	-0.08852	0.035462
36	0.24965	-0.47793	0.72903	-0.034255	0.037873
37	0.24465	-0.48961	0.73882	-0.084765	0.035408
38	0.24285	-0.49385	0.74192	-0.078358	0.036034
39	0.24645	-0.48538	0.73669	-0.065952	0.036288
40	0.23985	-0.50096	0.74484	0.056682	0.041187
41	0.23385	-0.51538	0.7483	-0.045458	0.036955
42	0.24305	-0.49337	0.74141	-0.035146	0.037374
43	0.25105	-0.47469	0.72383	0.036295	0.040473
44	0.24205	-0.49574	0.74288	-0.07316	0.036017

45	0.24165	-0.49669	0.74333	0.060037	0.041595
46	0.22444	-0.5385	0.75162	-0.021749	0.037611
47	0.24505	-0.48867	0.73815	-0.061062	0.036619
48	0.25045	-0.47608	0.72666	-0.079609	0.03597
49	0.22945	-0.52612	0.75021	-0.07047	0.035916
50	0.25765	-0.45958	0.69226	0.10042	0.043596
51	0.22805	-0.52957	0.75118	-0.068847	0.036115
52	0.23725	-0.50718	0.74699	0.029172	0.040222
53	0.24585	-0.48679	0.73707	-0.055732	0.037087
54	0.23205	-0.51976	0.74962	0.028092	0.039815
55	0.24185	-0.49621	0.74311	-0.067876	0.036159
56	0.23605	-0.51007	0.74755	-0.03268	0.03744
57	0.24745	-0.48305	0.73506	-0.065986	0.036269
58	0.23565	-0.51103	0.74791	-0.03591	0.037163
59	0.23705	-0.50766	0.74718	-0.054999	0.03679
60	0.22565	-0.53551	0.7514	-0.068158	0.035987
61	0.25685	-0.46139	0.70397	-0.024497	0.038613
62	0.25445	-0.46687	0.70945	-0.091866	0.035696
63	0.24045	-0.49954	0.7442	0.014088	0.039219
64	0.24425	-0.49055	0.74005	0.025128	0.039884
65	0.23665	-0.50862	0.74736	-0.074353	0.035832
66	0.25145	-0.47377	0.72274	0.090363	0.043662
67	0.25165	-0.47331	0.72028	-0.052191	0.03745
68	0.25725	-0.46048	0.69685	-0.070968	0.036447
69	0.25945	-0.4555	0.68669	-0.10783	0.035384
70	0.26025	-0.45369	0.67996	-0.091769	0.035449
71	0.28306	-0.40361	0.64837	0.1238	0.046354
72	0.24905	-0.47932	0.73214	-0.0048799	0.038981
73	0.24765	-0.48258	0.73417	-0.098883	0.035254
74	0.24365	-0.49196	0.74115	0.049505	0.041256
75	0.24905	-0.47932	0.73214	-0.013022	0.038373
76	0.22865	-0.52809	0.7508	-0.076267	0.035715
77	0.24965	-0.47793	0.72903	0.016685	0.040375
78	0.23885	-0.50335	0.74566	-0.045468	0.037082
79	0.22845	-0.52858	0.75099	0.0066267	0.038514
80	0.23785	-0.50574	0.74662	-0.055883	0.036491
81	0.23245	-0.51879	0.74925	-0.015127	0.038239
82	0.23925	-0.5024	0.74505	-0.0086841	0.038276
83	0.24965	-0.47793	0.72903	-0.078635	0.035972
84	0.24765	-0.48258	0.73417	-0.071493	0.0359
85	0.25145	-0.47377	0.72274	-0.030995	0.03791
86	0.25685	-0.46139	0.70397	-0.086638	0.035814
87	0.24645	-0.48538	0.73669	-0.075397	0.035912
88	0.23825	-0.50479	0.74605	-0.038006	0.037228
89	0.25545	-0.46459	0.70687	-0.081774	0.03583
90	0.23785	-0.50574	0.74662	0.028615	0.03996
91	0.23905	-0.50287	0.74526	-0.069438	0.035875
92	0.25085	-0.47515	0.72578	0.049199	0.041554
93	0.24545	-0.48773	0.7378	-0.06548	0.036091
94	0.58192	0.1436	0.41808	0.7583	0.091608
95	0.22324	-0.5415	0.75227	0.0039176	0.038439
96	0.24425	-0.49055	0.74005	-0.054445	0.036717
97	0.24005	-0.50049	0.74442	-0.076226	0.035939

98	0.24405	-0.49102	0.74034	0.028929	0.040208
99	0.25185	-0.47285	0.71887	-0.078675	0.035883
100	0.23765	-0.50622	0.74681	-0.074854	0.036104
101	0.29946	-0.36909	0.55931	0.22129	0.05192
102	0.24045	-0.49954	0.7442	-0.018008	0.037837
103	0.22905	-0.52711	0.7506	-0.027024	0.037852
104	0.28006	-0.41005	0.6603	0.16685	0.047737
105	0.23985	-0.50096	0.74484	-0.06991	0.035941
106	0.25265	-0.471	0.71733	0.073099	0.042348
107	0.29486	-0.37866	0.63123	0.19745	0.050444
108	0.22384	-0.54	0.75205	-0.036166	0.037194
109	0.24145	-0.49716	0.74377	-0.026229	0.037873
110	0.23225	-0.51927	0.74944	-0.046469	0.03664
111	0.29886	-0.37034	0.60659	0.20113	0.050016
112	0.25285	-0.47054	0.71567	0.054866	0.041666
113	0.23285	-0.51781	0.74887	-0.035798	0.037577

Table S3: Locus-specific component shared by all populations (α), level of differentiation (F_{ST}), q-value and posterior probability (log10 (PO) for loci under natural selection for the flat catchment *Rhodohypoxis baurii* var. *confecta* population. A total of 113 loci were scored from three primer combinations.

Locus No.	Probability	log10 (PO)	q-value	alpha	F_{ST}
1	0.178436	-0.66316	0.818897	0.034375	0.046585
2	0.15483	-0.73709	0.83485	-0.012191	0.042708
3	0.15823	-0.72590	0.83402	-0.0092317	0.042767
4	0.16703	-0.69782	0.82815	-0.0058623	0.043115
5	0.16343	-0.70916	0.83098	-0.011509	0.042695
6	0.16643	-0.69970	0.82880	-0.0044545	0.043428
7	0.17143	-0.68423	0.82502	-0.0091724	0.042943
8	0.17403	-0.67633	0.82276	0.023719	0.044842
9	0.15783	-0.72721	0.83426	-0.0098162	0.042873
10	0.15883	-0.72395	0.83380	-0.0062179	0.043231
11	0.15783	-0.72721	0.83426	-0.0031643	0.043124
12	0.17303	-0.67935	0.82376	-0.0050407	0.043281
13	0.16563	-0.70221	0.82975	-0.0041870	0.043125
14	0.16943	-0.69037	0.82660	-0.0039364	0.043190
15	0.16283	-0.71107	0.83166	-0.0095407	0.042930
16	0.15743	-0.72851	0.83433	-0.0077782	0.043106
17	0.16463	-0.70536	0.83022	-0.010728	0.042951
18	0.16143	-0.71555	0.83244	-0.0061209	0.042999
19	0.16543	-0.70284	0.83003	-0.0043142	0.043305
20	0.15723	-0.72917	0.83441	-0.0040587	0.043317
21	0.17223	-0.68179	0.82455	0.0020190	0.044028
22	0.16643	-0.69970	0.82880	-0.0032670	0.042973
23	0.15563	-0.73444	0.83475	-0.0099679	0.042333
24	0.16283	-0.71107	0.83166	-0.010635	0.042641
25	0.17544	-0.67211	0.82068	-0.015128	0.042846
26	0.18144	-0.65433	0.81756	0.021157	0.045977
27	0.16603	-0.70095	0.82935	-0.0045425	0.043184
28	0.16183	-0.71427	0.83228	-0.0082320	0.043214
29	0.16703	-0.69782	0.82815	0.0083444	0.044579
30	0.16563	-0.70221	0.82975	-0.0067998	0.043088
31	0.15983	-0.72070	0.83333	-0.0016391	0.043577
32	0.16423	-0.70663	0.83041	-0.010192	0.042890
33	0.17143	-0.68423	0.82502	-0.0065533	0.043140
34	0.16263	-0.71171	0.83182	0.00074473	0.043725
35	0.17043	-0.68729	0.82597	-0.0018296	0.043981
36	0.17123	-0.68484	0.82523	-0.0042633	0.043452
37	0.16123	-0.71619	0.83266	-0.019336	0.042348
38	0.17784	-0.66494	0.81971	-0.0065283	0.043393

39	0.17023	-0.68791	0.82613	-0.0039251	0.043310
40	0.15883	-0.72395	0.83380	-0.0059880	0.043130
41	0.16563	-0.70221	0.82975	-0.0062369	0.043078
42	0.15663	-0.73114	0.83450	-0.013160	0.042425
43	0.16243	-0.71235	0.83197	-0.015236	0.043111
44	0.17103	-0.68545	0.82543	-0.0029201	0.043182
45	0.16203	-0.71363	0.83205	-0.0047849	0.043172
46	0.16183	-0.71427	0.83228	-0.013190	0.042796
47	0.17323	-0.67875	0.82347	-0.010123	0.043288
48	0.16763	-0.69595	0.82757	-0.017662	0.042583
49	0.16363	-0.70853	0.83070	-0.0046680	0.043160
50	0.18344	-0.64850	0.81656	-0.017448	0.042981
51	0.16623	-0.70033	0.82903	-0.0068682	0.043168
52	0.15983	-0.72070	0.83333	0.0012754	0.043599
53	0.16683	-0.69845	0.82829	-0.0077173	0.043189
54	0.16103	-0.71683	0.83288	-0.016493	0.042459
55	0.15903	-0.72330	0.83356	-0.0072085	0.043242
56	0.16543	-0.70284	0.83003	-0.010092	0.042911
57	0.15883	-0.72395	0.83380	0.0021571	0.043359
58	0.15783	-0.72721	0.83426	-0.010060	0.042665
59	0.16023	-0.71941	0.83303	0.0020952	0.043392
60	0.16603	-0.70095	0.82935	-0.0073208	0.043318
61	0.16183	-0.71427	0.83228	-0.0023251	0.043244
62	0.16303	-0.71043	0.83133	0.0078079	0.044292
63	0.16123	-0.71619	0.83266	-0.0032389	0.043366
64	0.16543	-0.70284	0.83003	-0.015305	0.042858
65	0.16603	-0.70095	0.82935	-0.0092281	0.043058
66	0.15963	-0.72135	0.83341	-0.0064078	0.043072
67	0.16743	-0.69658	0.82772	0.031690	0.046072
68	0.15583	-0.73377	0.83467	0.00066670	0.043490
69	0.17443	-0.67512	0.82236	-0.00020842	0.043787
70	0.17483	-0.67391	0.82191	0.0010105	0.044117
71	0.17343	-0.67814	0.82314	0.012743	0.044742
72	0.17083	-0.68607	0.82562	-0.015277	0.042853
73	0.16763	-0.69595	0.82757	-0.0088977	0.043049
74	0.16923	-0.69099	0.82676	-0.0037330	0.043370
75	0.15423	-0.73908	0.83494	-0.0080548	0.043177
76	0.16303	-0.71043	0.83133	-0.00087902	0.043771
77	0.17223	-0.68179	0.82455	0.048176	0.047076
78	0.16663	-0.69907	0.82842	-0.0057369	0.043406
79	0.16323	-0.70980	0.83116	-0.013245	0.043037
80	0.16423	-0.70663	0.83041	-0.0039678	0.043203
81	0.16983	-0.68914	0.82645	-0.0021141	0.043632
82	0.15863	-0.72460	0.83387	-0.0016647	0.043401
83	0.16103	-0.71683	0.83288	-0.0066316	0.042916
84	0.16343	-0.70916	0.83098	0.0048027	0.043718

85	0.17524	-0.67271	0.82136	0.010224	0.044274
86	0.16243	-0.71235	0.83197	-0.00095537	0.043129
87	0.16123	-0.71619	0.83266	-0.0021667	0.043554
88	0.16723	-0.69720	0.82787	-0.013223	0.042553
89	0.17003	-0.68852	0.82629	-0.00076117	0.043963
90	0.16343	-0.70916	0.83098	-0.0079891	0.042860
91	0.16003	-0.72006	0.83310	-0.0062679	0.043030
92	0.16383	-0.70789	0.83060	-0.0074286	0.043017
93	0.16263	-0.71171	0.83182	-0.0091594	0.043334
94	0.16643	-0.69970	0.82880	-0.0040882	0.043175
95	0.16083	-0.71748	0.83295	-0.0060582	0.043035
96	0.16403	-0.70726	0.83051	-0.013398	0.042868
97	0.16823	-0.69409	0.82725	-0.0025920	0.043664
98	0.16823	-0.69409	0.82725	-0.0054767	0.043487
99	0.15983	-0.72070	0.83333	-0.0046543	0.042992
100	0.16483	-0.70473	0.83012	0.010637	0.044471
101	0.16283	-0.71107	0.83166	-0.010474	0.042935
102	0.16323	-0.70980	0.83116	-0.012298	0.042622
103	0.16103	-0.71683	0.83288	0.013471	0.044173
104	0.16623	-0.70033	0.82903	-0.0052663	0.043190
105	0.16143	-0.71555	0.83244	0.00065411	0.043733
106	0.15843	-0.72525	0.83395	-0.0065376	0.042876
107	0.17063	-0.68668	0.82579	-0.015414	0.043018
108	0.17243	-0.68118	0.82406	-0.0099152	0.043551
109	0.16843	-0.69347	0.82693	0.0084537	0.044088
110	0.16563	-0.70221	0.82975	-0.0017275	0.043243
111	0.15643	-0.73180	0.83458	-0.0024210	0.043193
112	0.15903	-0.72330	0.83356	-0.011268	0.042688
113	0.16283	-0.71107	0.83166	0.00062430	0.043793

Table S4: Locus-specific component shared by all populations (α), level of differentiation (F_{ST}), q-value and posterior probability (log10 (PO) for loci under natural selection for the Witsieshoek lodge *Rhodohypoxis baurii* var. *confecta* population. A total of 113 loci were scored from three primer combinations.

Locus No.	Probability	log10 (PO)	q-value	alpha	F_{ST}
1	0.161232	-0.71619	0.831854	0.0015910	0.073753
2	0.16363	-0.70853	0.83029	-0.013953	0.072582
3	0.16143	-0.71555	0.83161	-0.018556	0.072288
4	0.16223	-0.71299	0.83137	-0.0074267	0.072915
5	0.15963	-0.72135	0.83263	-0.014785	0.072495
6	0.16543	-0.70284	0.82895	-0.0044935	0.074385
7	0.16223	-0.71299	0.83137	-0.020450	0.071805
8	0.14723	-0.76284	0.83398	-0.013621	0.072236
9	0.17243	-0.68118	0.82268	-0.013670	0.072810
10	0.16703	-0.69782	0.82680	-0.023711	0.072517
11	0.17143	-0.68423	0.82321	-0.016656	0.072370
12	0.16943	-0.69037	0.82504	0.0088381	0.075005
13	0.16043	-0.71876	0.83239	-0.0076688	0.073219
14	0.15863	-0.72460	0.83309	-0.0078479	0.073416
15	0.16503	-0.70410	0.82916	-0.010678	0.073114
16	0.17023	-0.68791	0.82420	-0.0019037	0.074368
17	0.16603	-0.70095	0.82829	-0.0019221	0.073966
18	0.16783	-0.69533	0.82648	-0.0047424	0.073646
19	0.18744	-0.63700	0.81256	0.044284	0.079299
20	0.17924	-0.66079	0.81870	0.031803	0.077720
21	0.17964	-0.65961	0.81828	0.037556	0.078246
22	0.16423	-0.70663	0.82993	-0.012803	0.073384
23	0.16443	-0.70599	0.82965	-0.0031852	0.073620
24	0.16403	-0.70726	0.83002	-0.0079859	0.072704
25	0.16283	-0.71107	0.83088	-0.0024379	0.073495
26	0.17624	-0.66971	0.82085	0.034381	0.078005
27	0.16983	-0.68914	0.82484	-0.0032132	0.074488
28	0.15943	-0.72200	0.83278	-0.012811	0.072753
29	0.16343	-0.70916	0.83046	-0.021340	0.072481
30	0.16583	-0.70158	0.82841	-0.0010264	0.074093
31	0.16163	-0.71491	0.83153	-0.018867	0.072110
32	0.17684	-0.66792	0.81990	-0.00013258	0.074478
33	0.17463	-0.67451	0.82186	-0.017609	0.073021
34	0.16463	-0.70536	0.82946	-0.0091641	0.073296
35	0.16843	-0.69347	0.82581	-0.013137	0.073318
36	0.17584	-0.67091	0.82110	0.040937	0.079021
37	0.15923	-0.72265	0.83294	-0.014428	0.072521
38	0.15743	-0.72851	0.83318	-0.012521	0.072386

39	0.15703	-0.72982	0.83336	-0.015727	0.073363
40	0.16383	-0.70789	0.83020	-0.013788	0.072828
41	0.16083	-0.71748	0.83216	-0.011511	0.073028
42	0.16423	-0.70663	0.82993	-0.011066	0.072847
43	0.16663	-0.69907	0.82754	-0.014204	0.072945
44	0.17023	-0.68791	0.82420	-0.020336	0.072160
45	0.16023	-0.71941	0.83247	-0.0094081	0.073071
46	0.16383	-0.70789	0.83020	-0.010456	0.072708
47	0.16303	-0.71043	0.83080	-0.0055290	0.073608
48	0.16983	-0.68914	0.82484	-0.015442	0.072642
49	0.16603	-0.70095	0.82829	-0.012496	0.072940
50	0.16623	-0.70033	0.82793	0.0090833	0.075346
51	0.16103	-0.71683	0.83201	-0.0059701	0.072973
52	0.16303	-0.71043	0.83080	-0.00090761	0.073642
53	0.17524	-0.67271	0.82136	0.038690	0.078163
54	0.16303	-0.71043	0.83080	-0.012323	0.072395
55	0.15903	-0.72330	0.83301	-0.021898	0.072281
56	0.16643	-0.69970	0.82767	-0.013981	0.073420
57	0.15943	-0.72200	0.83278	-0.017154	0.072236
58	0.16003	-0.72006	0.83255	-0.012884	0.072594
59	0.17463	-0.67451	0.82186	-0.0048685	0.073770
60	0.15343	-0.74175	0.83367	-0.011955	0.072258
61	0.18104	-0.65550	0.81576	0.041572	0.079169
62	0.16843	-0.69347	0.82581	-0.0019197	0.073597
63	0.16443	-0.70599	0.82965	0.00019999	0.074300
64	0.16683	-0.69845	0.82711	-0.011143	0.073285
65	0.16543	-0.70284	0.82895	0.0039978	0.074484
66	0.18084	-0.65609	0.81690	0.040923	0.078375
67	0.16623	-0.70033	0.82793	-0.0063479	0.073183
68	0.17103	-0.68545	0.82347	0.036361	0.078482
69	0.17724	-0.66672	0.81949	0.044516	0.079234
70	0.17203	-0.68240	0.82294	-0.0027625	0.073967
71	0.16483	-0.70473	0.82936	-0.025587	0.071991
72	0.16063	-0.71812	0.83224	-0.010982	0.072594
73	0.16503	-0.70410	0.82916	-0.0027236	0.073935
74	0.17964	-0.65961	0.81828	0.037803	0.078257
75	0.16543	-0.70284	0.82895	-0.0070484	0.073767
76	0.17383	-0.67693	0.82212	0.0038359	0.074494
77	0.16563	-0.70221	0.82863	-0.0050730	0.073471
78	0.16743	-0.69658	0.82664	-0.021278	0.072223
79	0.16223	-0.71299	0.83137	-0.013666	0.072796
80	0.15623	-0.73246	0.83346	-0.019894	0.072037
81	0.15723	-0.72917	0.83327	-0.010865	0.072828
82	0.15123	-0.74915	0.83381	-0.0074291	0.072832
83	0.16483	-0.70473	0.82936	-0.015552	0.072844
84	0.17083	-0.68607	0.82372	-0.0038026	0.073996

85	0.16663	-0.69907	0.82754	-0.0077121	0.073021
86	0.16123	-0.71619	0.83185	-0.022576	0.072280
87	0.16263	-0.71171	0.83113	-0.013118	0.072532
88	0.16083	-0.71748	0.83216	-0.014338	0.072509
89	0.16423	-0.70663	0.82993	-0.00045086	0.073807
90	0.16343	-0.70916	0.83046	-0.0026021	0.073594
91	0.16043	-0.71876	0.83239	-0.010910	0.073111
92	0.17904	-0.66138	0.81902	0.028774	0.077172
93	0.16823	-0.69409	0.82615	-0.0061038	0.073576
94	0.16843	-0.69347	0.82581	-0.011699	0.072902
95	0.16563	-0.70221	0.82863	-0.017063	0.072945
96	0.16903	-0.69161	0.82524	-0.024284	0.071833
97	0.15923	-0.72265	0.83294	-0.017943	0.072207
98	0.16263	-0.71171	0.83113	0.0064957	0.074747
99	0.16663	-0.69907	0.82754	-0.0039191	0.073311
100	0.17624	-0.66971	0.82085	0.025510	0.077405
101	0.17644	-0.66911	0.82026	0.037444	0.078445
102	0.17263	-0.68057	0.82241	-0.0027942	0.074154
103	0.16163	-0.71491	0.83153	-0.012083	0.072778
104	0.16123	-0.71619	0.83185	-0.019037	0.072015
105	0.16783	-0.69533	0.82648	-0.0092144	0.073143
106	0.16983	-0.68914	0.82484	-0.0015400	0.073591
107	0.16823	-0.69409	0.82615	0.039185	0.077965
108	0.16103	-0.71683	0.83201	-0.0067171	0.073335
109	0.16323	-0.70980	0.83055	-0.017065	0.072600
110	0.15563	-0.73444	0.83356	-0.017593	0.072413
111	0.16263	-0.71171	0.83113	-0.0089727	0.073373
112	0.16603	-0.70095	0.82829	-0.0080788	0.073158
113	0.16683	-0.69845	0.82711	0.018501	0.075507

Table S5: Locus-specific component shared by all populations (α), level of differentiation (F_{ST}), q-value and posterior probability (log10 (PO) for loci under natural selection for the Sentinel peak *Rhodohypoxis baurii* var. *confecta* population. A total of 113 loci were scored from three primer combinations.

Locus No.	Probability	log10 (PO)	q-value	alpha	F_{ST}
1	0.164633	-0.705361	0.828867	0.0037813	0.11360
2	0.15883	-0.72395	0.83155	-0.026969	0.10971
3	0.17123	-0.68484	0.82357	-0.014815	0.11072
4	0.15863	-0.72460	0.83166	-0.0085074	0.11164
5	0.15283	-0.74376	0.83421	-0.025862	0.10907
6	0.16563	-0.70221	0.82806	-0.00039586	0.11225
7	0.15063	-0.75119	0.83460	-0.016375	0.11061
8	0.16243	-0.71235	0.82994	-0.016581	0.11103
9	0.17504	-0.67331	0.81826	-0.014942	0.11097
10	0.15903	-0.72330	0.83144	-0.016680	0.11063
11	0.15803	-0.72655	0.83200	-0.024489	0.10947
12	0.16523	-0.70347	0.82841	-0.0059263	0.11190
13	0.15743	-0.72851	0.83244	-0.016833	0.11059
14	0.16523	-0.70347	0.82841	-0.0025500	0.11310
15	0.16143	-0.71555	0.83067	-0.017378	0.11086
16	0.17003	-0.68852	0.82481	-0.024243	0.11016
17	0.17143	-0.68423	0.82290	0.024509	0.11623
18	0.16663	-0.69907	0.82689	0.0074255	0.11396
19	0.17103	-0.68545	0.82413	0.0039361	0.11342
20	0.16023	-0.71941	0.83099	-0.0018931	0.11283
21	0.16663	-0.69907	0.82689	-0.0034491	0.11233
22	0.17163	-0.68362	0.82235	0.016174	0.11502
23	0.16383	-0.70789	0.82940	-0.012377	0.11122
24	0.17163	-0.68362	0.82235	-0.0082568	0.11144
25	0.17343	-0.67814	0.81890	0.023022	0.11576
26	0.17003	-0.68852	0.82481	0.0060772	0.11387
27	0.16083	-0.71748	0.83088	-0.0060068	0.11203
28	0.15583	-0.73377	0.83302	-0.030700	0.10892
29	0.15823	-0.72590	0.83189	-0.024069	0.10946
30	0.16263	-0.71171	0.82983	-0.012328	0.11095
31	0.17103	-0.68545	0.82413	-0.034194	0.10923
32	0.16463	-0.70536	0.82887	-0.014711	0.11127
33	0.16743	-0.69658	0.82591	-0.011035	0.11166
34	0.16223	-0.71299	0.83026	-0.021665	0.11004
35	0.16643	-0.69970	0.82731	-0.0012535	0.11291
36	0.17063	-0.68668	0.82430	-0.0042673	0.11347
37	0.15323	-0.74242	0.83397	-0.019842	0.11003
38	0.15343	-0.74175	0.83373	-0.019017	0.11005

39	0.16803	-0.69471	0.82556	-0.025824	0.11007
40	0.16443	-0.70599	0.82908	-0.012209	0.11164
41	0.16003	-0.72006	0.83121	-0.018796	0.10990
42	0.15323	-0.74242	0.83397	-0.017741	0.11065
43	0.15463	-0.73775	0.83336	-0.020265	0.10961
44	0.17123	-0.68484	0.82357	-0.024461	0.10982
45	0.16463	-0.70536	0.82887	-0.023638	0.10955
46	0.15583	-0.73377	0.83302	-0.0086229	0.11108
47	0.16223	-0.71299	0.83026	-0.023682	0.11014
48	0.15563	-0.73444	0.83313	-0.0090825	0.11094
49	0.15523	-0.73576	0.83324	-0.0097405	0.11085
50	0.17123	-0.68484	0.82357	0.025009	0.11635
51	0.15283	-0.74376	0.83421	-0.020615	0.10973
52	0.18504	-0.64388	0.81176	0.069385	0.12190
53	0.16563	-0.70221	0.82806	-0.014409	0.11100
54	0.16523	-0.70347	0.82841	-0.015206	0.11103
55	0.15623	-0.73246	0.83279	-0.017297	0.11065
56	0.16203	-0.71363	0.83046	-0.012424	0.11171
57	0.15783	-0.72721	0.83222	-0.023327	0.10943
58	0.16103	-0.71683	0.83078	-0.028863	0.10924
59	0.16763	-0.69595	0.82574	0.024977	0.11597
60	0.15383	-0.74041	0.83360	-0.021599	0.11007
61	0.17243	-0.68118	0.82053	-0.011169	0.11195
62	0.17203	-0.68240	0.82135	-0.00071972	0.11354
63	0.18244	-0.65141	0.81446	0.065752	0.12085
64	0.16383	-0.70789	0.82940	0.020369	0.11500
65	0.17664	-0.66851	0.81702	0.0099940	0.11399
66	0.17964	-0.65961	0.81576	0.069179	0.12197
67	0.16443	-0.70599	0.82908	-0.0085740	0.11207
68	0.17103	-0.68545	0.82413	0.010668	0.11445
69	0.17203	-0.68240	0.82135	0.0038217	0.11356
70	0.16143	-0.71555	0.83067	-0.0083270	0.11177
71	0.16683	-0.69845	0.82659	-0.025508	0.10998
72	0.16343	-0.70916	0.82951	-0.020634	0.11023
73	0.15903	-0.72330	0.83144	-0.0086372	0.11152
74	0.17243	-0.68118	0.82053	0.034164	0.11722
75	0.16563	-0.70221	0.82806	-0.020411	0.11075
76	0.15823	-0.72590	0.83189	-0.021757	0.11057
77	0.18284	-0.65025	0.81384	0.066877	0.12188
78	0.16463	-0.70536	0.82887	-0.016131	0.11018
79	0.16623	-0.70033	0.82769	-0.0075761	0.11223
80	0.15083	-0.75051	0.83447	-0.025365	0.10880
81	0.17924	-0.66079	0.81632	0.015055	0.11517
82	0.15703	-0.72982	0.83255	-0.011544	0.11057
83	0.15243	-0.74510	0.83433	-0.012941	0.11082
84	0.17604	-0.67031	0.81765	-0.015107	0.11111

85	0.16623	-0.70033	0.82769	-0.0090140	0.11134
86	0.15443	-0.73841	0.83348	-0.028049	0.10897
87	0.16803	-0.69471	0.82556	-0.0062337	0.11206
88	0.16643	-0.69970	0.82731	-0.013028	0.11067
89	0.16623	-0.70033	0.82769	0.011720	0.11464
90	0.16383	-0.70789	0.82940	0.0056265	0.11319
91	0.15043	-0.75186	0.83473	-0.0055559	0.11112
92	0.16683	-0.69845	0.82659	-0.012341	0.11124
93	0.16003	-0.72006	0.83121	0.0034238	0.11317
94	0.16643	-0.69970	0.82731	0.010958	0.11429
95	0.16203	-0.71363	0.83046	-0.0080682	0.11176
96	0.16223	-0.71299	0.83026	-0.017655	0.11053
97	0.17043	-0.68729	0.82447	-0.0031087	0.11203
98	0.18884	-0.63302	0.81016	0.062916	0.12129
99	0.16683	-0.69845	0.82659	-0.0030243	0.11255
100	0.16863	-0.69285	0.82500	0.011423	0.11512
101	0.19084	-0.62737	0.80916	0.086878	0.12502
102	0.18324	-0.64908	0.81301	0.021120	0.11593
103	0.16823	-0.69409	0.82519	0.013331	0.11464
104	0.15783	-0.72721	0.83222	-0.0059254	0.11169
105	0.17143	-0.68423	0.82290	0.0012347	0.11298
106	0.15623	-0.73246	0.83279	-0.0092336	0.11130
107	0.18104	-0.65550	0.81511	0.064505	0.12133
108	0.16283	-0.71107	0.82973	-0.019163	0.11097
109	0.16323	-0.70980	0.82962	-0.014668	0.11082
110	0.15743	-0.72851	0.83244	-0.019897	0.11032
111	0.17163	-0.68362	0.82235	0.024443	0.11608
112	0.16683	-0.69845	0.82659	-0.015506	0.11106
113	0.17243	-0.68118	0.82053	-0.0092121	0.11170