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Colours of confetti: the role of ABP genes and environmental variables in flower colour polymorphisms of *Rhodohypoxis baurii* var. *confecta*

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727181

A dissertation submitted to the Faculty of Science,
University of the Witwatersrand,
Johannesburg, South Africa
in fulfilment of the requirements for the degree of
Master of Science

Signed on 27 February 2019 in Johannesburg

"From so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved"
Charles Darwin

for my parents, Martina and Tony Gardiner,
for their support and guidance.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree if 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.



Courtney Elizabeth Campbell Gardiner

27th day of February 2019

Supervisor (MSc):

Dr Kelsey L. Glennon (*University of the Witwatersrand*)

PREFACE

This dissertation consists of four chapters: a general introduction, two results chapters, and a general conclusion with recommendations for future work. The two research chapters have each been prepared with the intention to publish and have their own abstracts, and reference sections. There is some repetition between chapters, as the two results chapters are intended to stand-alone and omitting the duplicated information would render the chapters incomplete.

Chapter Two was presented at the 44th Annual Conference of South African Association of Botanists (SAAB) in Pretoria, Gauteng during 9-12 January 2018 and the abstract from this presentation was published in the South African Journal of Botany in April 2019. A poster displaying this work was presented at the II Joint Congress on Evolutionary Biology in Montpellier, France during 18-22 August 2018. In addition, the chapter was adapted and submitted to the International Journal of Plant Science on 3 February 2019 and at the time of submission is under review.

Chapter Three was presented at the 45th Annual South African Association of Botanists (SAAB), African Mycological Association (AMA), and South African Society for Systematic Biology (SASSB) Congress in Johannesburg, Gauteng during 8-11 January 2019 and the abstract from this presentation will be published in the South African Journal of Botany in late 2019.

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ABSTRACT

The study of flower colour is a particularly valuable approach to investigating fundamental evolutionary questions such as the maintenance of variation within species, the role of natural selection and genetic drift in maintaining polymorphisms, and how such polymorphisms contribute to biodiversity. Flower colour is a phenotype that is easily measured and it provides a strong indicator of the outcomes of selection pressures. It is both driven and maintained by either non- pollinator mediated selection agents, pollinator selection agents, or a combination of both.

The overall aim of this study was to investigate the maintenance of flower colour using *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard and Burtt as a study system. This involved understanding the genetic mechanisms that regulate flower colour and examining the environmental variables that drive this trait variation across populations. Molecular and ecological methods were used in combination to understand flower colour by investigating the genes responsible, as well as the environmental selection pressures acting on this phenotype.

Rhodoypoxis baurii var. *confecta* is a Drakensberg near-endemic species that has naturally occurring populations of magenta, pink, and white flower colour morphs. These flower colour morphs are discrete variants that co-exist in single populations and are thus considered to be true polymorphisms. Three populations of *R. baurii* var. *confecta* that occur in Sentinel Peak in the Royal Natal National Park, South Africa were studied.

Non-pollinator mediated selection agents were investigated as potential drivers of flower variation in *R. baurii* var. *confecta*. The frequency of unpigmented (white) and pigmented (pink/magenta) flower colour morphs was measured over the flowering season. No pollinator visitation was observed at populations of *R. baurii* var. *confecta* and therefore pollinator-mediated selection was excluded. The extent to which non-pollinator selection agents drive this intra-population variation was investigated by measuring the change in soil moisture, temperature, and precipitation over the flowering season. One population shifted from a greater proportion of unpigmented morphs at the beginning of the flowering season to a relatively greater proportion of pigmented morphs at the end of the season. In this population, a positive correlation was found between the proportion of pigmented morphs and soil moisture. This suggests that the accumulation of water in the soil promotes the production of pigmented flowers.

The anthocyanin biosynthetic pathway (ABP) is responsible for the production of pigment in *R. baurii* var. *confecta*. The ABP is pleiotropic and in addition to pigment production is responsible for traits related to plant physiology and environmental stress response. Consequently, the ABP is cited as being well conserved among angiosperm species. The presence of expression in four ABP genes (*CHI*, *F3H, F3'H,* and *F3'5'H*) was tested for and compared between unpigmented and pigmented *R. baurii* var. *confecta* flowers. As there is no sequence data for *Rhodohypoxis* or any Hypoxidaceae species, primers for this molecular work were designed using closely related monocotyledon ABP gene sequences. Sequencing results showed that the amplified PCR products were not the targeted ABP genes. These results suggested that the designed primers were non-specific.

The similarity of the four ABP genes within angiosperm species was investigated. All available complete sequences of the four regions of interest were aligned and sequence similarity was quantified. The results from this alignment indicate that the four investigated ABP gene sequences are polymorphic and are likely not well conserved within angiosperm species as a whole and, to some extent, within monocotyledon and dicotyledon species respectively. This suggests that the basic structure of the ABP is well conserved amongst angiosperm species however, the gene sequences that make up the pathway are polymorphic and less well conserved.

The study highlights the importance of non-pollinator mediated selection on the presence and maintenance of flower colour polymorphisms. In addition, it provides some insight on the ABP and its conservation amongst angiosperm species. It also contributes to understanding how flower colour polymorphisms are maintained in natural populations both on a molecular and ecological level and is the foundational work in understanding the polymorphisms of *R. baurii* var. *confecta*.

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CHAPTER ONE

GENERAL INTRODUCTION

Natural selection is one of the key mechanisms that drives the process of evolution (Darwin, 1859; Dobzhansky, 1937). Variation within natural populations allows for the selection of the fittest and favours diversity in populations under different conditions. Certain individuals can be better adapted to local environmental conditions and are reproductively more successful than others (Darwin; 1959; Sober, 1984; Bell, 1997). This selection eventually results in distinct populations that differ substantially to other populations of the same species (Mayr, 1947; 1963). The divergence, whether the product of geographical or reproductive isolation, ultimately results in speciation and an increase in biodiversity (Mayr, 1947; Otte and Endler, 1989; Coyne and Orr, 2004).

Polymorphisms are examples of evolutionary divergence in species as a result of selection pressures (Gray and McKinnon, 2007). They are defined as the occurrence and maintenance of two or more distinct forms of a species in the same habitat (Ford, 1940; Huxley, 1955). Polymorphisms result in increased genetic diversity and as a result are believed to decrease vulnerability to environmental change and to promote the ecological and evolutionary success of the species and populations (Forsman *et al*. 2008; Forsman, 2015). Forsman (2015) has found that highly variable, polymorphic populations show decreased fluctuations in population size, are more likely to have establishment success, large distribution ranges and are less prone to extinction.

Flower colour polymorphisms

Variation in flower colour occurs at a number of different taxonomic levels (Smith and Goldberg, 2015), ranging from family to species level (e.g., Ellis and Field, 2016; Quattrocchio *et al*. 1999; Schwinn *et al*. 2006; Whittall *et al*. 2006), within species at the population level (e.g., Schemske and Bradshaw, 1999; Schemske and Bierzychudek, 2001; Carlson and Holsinger, 2015) and even within individual flowers (e.g., Feild *et al*. 2001; Farzad *et al*. 2002; Willmer, 2009). While flower colour variations are common amongst angiosperms, stable and genetically based intra-population and intra-specific polymorphisms are much less common (Kay, 1978). Flower colour polymorphisms occur at a species level and are characterised by two or more discrete variants of a species (Narbona *et al.* 2018). As a result, flower colour variation occurring at higher taxonomic levels (e.g., genus, class, or family level) is not considered to be a true polymorphism. Furthermore, flower colour changes occurring in individual flowers as a result of interaction with pollinators (e.g., *Desmodium setigerum*; Willmer, 2009) or developmentally transient pigment production (e.g., *Viola cornuta*, 'Yesterday, Today, Tomorrow'; Farzad *et al*. 2002) are not classified as flower colour polymorphisms (Chalker-Scott, 1999; Feild *et al.* 2001).

Flower colour polymorphisms have the potential to increase biodiversity by contributing to plant phenotypic and species diversity (Rausher, 2008; Ellis and Field, 2016) and are often the most noticeable example of intra-population and intra-specific genetic variation in plants. Consequently, flower colour is an excellent model for studying polymorphisms in natural populations. Studying such polymorphisms can provide insight into a variety of key evolutionary questions, such as how variation is

maintained within a species, what roles natural selection and genetic drift play in maintaining polymorphisms, and ultimately how such polymorphisms contribute to biodiversity (Clegg and Durbin, 2000; Gigord *et al.* 2001; Irwin, 2003; Schemske and Bierzychudek, 2007; Coburn *et al*. 2015; Ellis and Field, 2016).

Anthocyanin Biosynthetic Pathway

Three biochemical pathways are responsible for the diversity of flower colour observed in angiosperms. These biochemical pathways are responsible for the production of betalain (only found in Caryophyllales), carotenoid, and anthocyanin pigments. These pathways have resulted in the convergent evolution of similar flower colours across many flowering plant lineages and are highly conserved across angiosperm species (Holton and Cornish, 1995; Winkel-Shirley, 2002; Grotewold, 2006).

Anthocyanins are the most common pigments responsible for flower colour and are produced by the anthocyanin biosynthetic pathway (ABP; Tanaka *et al*. 2008). Anthocyanins give rise to a variety of flower colours ranging from pale yellow to blue to red (Tanaka *et al.* 2008). Plants containing anthocyanins are common and widely distributed throughout angiosperms, for instance, they have been documented to occur in approximately 20% of British flora (Warren and Mackenzie, 2001). Since anthocyanin pigments are so common, the genetics and biochemistry of the ABP pathway has been well described (Harborne, 1988; Stafford, 1990; Forkmann, 1991; Mol *et al*. 1993; Grotewold, 2006).

The ABP is responsible for such a wide variety of flower colours through alterations in pigmentation intensity (concentration of pigment), hue (types of pigments

and co-pigments) (Hopkins and Rausher, 2011; Sobel and Streisfield, 2013) and other physical and chemical cell factors (Miller, 2011). The pathway produces three primary types of pigments, namely pelargonidin, cyanidin, and delphinidin, which are produced in different combinations and concentrations to produce distinctive flower colours. Pelargonidins result in orange colours, delphinidins in blue hues, and cyanidins in reddish-purple colours (Tanaka *et al*. 2008).

In addition to the production of anthocyanins, the ABP also produces other flavonoids including flavones, flavonones, and flavonols (Koes *et al.* 1994; Chalker-Scott, 1999; Grotewold, 2006; Ryan *et al*. 2002; Tanaka *et al.* 2008). These compounds play an important role in environmental stress response and plant physiology, including plant disease defence, pollen viability, auxin transport, microbial interactions, trichome density, root hair formation, cell morphology, vacuolar size, and UV protection (Koes *et al*. 1994; Mol *et al.* 1993; Walker *et al*. 1999; Spelt *et al*. 2002; Winkel-Shirley, 2002; Morita *et al*. 2006). The ABP is therefore pleiotropic as the genes in the pathway influence multiple unrelated phenotypic traits and it is for this reason that the ABP is considered to be well conserved across angiosperm species (Quattrocchio *et al.* 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al.* 2014). The first three genes in the ABP pathway (*CHS-CHI-F3H*) evolved when land plants first emerged in order to provide protection against UV radiation (Rausher, 2008). As a result, the pathway has existed long before the evolution of angiosperms, and anthocyanin pigments were produced much later in evolutionary history. Consequently, the ABP pathway did not initially emerge in evolutionary history

to attract pollinators, but rather evolved in response to environmental stresses (Hanley *et al.* 2009; Whittall and Carlson, 2009).

The eight genes of the ABP are divided into early biosynthetic genes (EBGs; *CHS*, *CHI*, and *F3H*) and the late biosynthetic genes (LBGs; *F3'H*, *F3'5'H*, *DFR*, *ANS*, and *UF3GT*)(Liu *et al.* 2018). The EBGs (*CHS*, *CHI*, and *F3H*) are found at the beginning of the ABP and result in the production of flavonoids including flavones, flavonones, and flavonols (Koes *et al.* 1994; Chalker-Scott, 1999; Grotewold, 2006; Ryan *et al.* 2002; Tanaka *et al.* 2008). These EBGs occur upstream of the LBGs and provide the precursor for all classes of flavonoids. The expression of *F3H*, *F3'H*, and *F3'5'H,* which occur further downstream, results in the production of three different types of anthocyanin pigments. These pigments are: pelargonidin, cyanidin, and delphinidin (Holton and Cornish, 1995). The presence of expression and levels of expression of these genes dictate the intensity (concentration of pigment) and hue (types of pigments and co-pigments) of flower colour acorss angiosperm species.

Selection agents acting on flower colour

Colour variation is shaped by selection; however, the agents behind this selection are diverse and have been debated (Strauss and Whittall, 2006; Schemske and Bierzychudek, 2007; Streisfield and Kohn, 2007; Rausher, 2008). Two possible selection agent hypotheses have been put forward: pollinator-mediated selection (PMS) and non-pollinator mediated selection (NPMS). Plant fitness can be directly related to both pollinator preferences as well as non-pollinator related traits (e.g., Levin and Brack, 1995; Gómez, 2003; Coberly and Rausher, 2003; Strauss *et al.* 2004). Much work is needed to better understand the relative strength of pollinator versus non-pollinator

mediated selection on flower colour polymorphisms (Strauss and Whittall, 2006). For instance, combinations of biotic and abiotic factors may determine whether a phenotype is adapted to a particular environment and these factors may collectively impact survival and reproductive success (Strauss and Whittall, 2006). Consequently, it may be difficult to isolate any single selecting agent.

A. Pollinator-mediated selection

It is conventionally thought that pollinators are the primary driver of flower colour variation in angiosperms, as one of the fundamental functions of flower pigmentation is to attract pollinators. Flower colour attracts pollinators through visual colour displays or the heat produced by pigments (Mol *et al*. 1998; Miller, 2011). Different pollinators perceive flower colour differently and certain groups of pollinators have been found to favour specific floral characteristics (Grant, 1949; Stebbins, 1974; Meléndez-Ackerman and Campbell, 1998; Hodges *et al*. 2002; Fenster *et al*. 2004). These trends are known as pollinator syndromes, which are defined as generalized groups of floral traits that have been associated with specific pollinator agents (Fenster *et al*. 2004).

Consequently, pollinators are discerning when it comes to flower colour and changes in flower colour can have a considerable impact on the spectrum of pollinators that come into contact with a plant. Therefore floral pigments (e.g., anthocyanins) are a strong causal factor contributing to the reproductive success of the plant, as the occurrence of flower colour polymorphisms can potentially reduce gene flow between different coloured morphs, and this could promote speciation (Schemske and Bradshaw,

1999; Hodges *et al*. 2002; Bradshaw and Schemske, 2003). Stanton (1987) for instance, found that white morphs of *Raphanus sativus* L. were preferred by honeybee pollinators whereas, Waser and Price (1981) found that white morphs of *Delphinium nelsonii* L. were visited less by bumblebees and hummingbirds compared to blue morphs in the same population. Mutational changes that result in flower colour polymorphisms within a population can thus have potentially profound effects on diversity. As a result, pollinators could potentially drive the diversity of flower colour in many plant species (Waser and Price, 1981; Stanton, 1987; Quattrocchio *et al*. 1999; Fenster *et al*. 2004; Waser and Campbell, 2004).

An unusual example of how pollinators drive flower colour variation is found in the rewardless Elderflower orchid, *Dactylorhiza sambucina* (L.) Soó. This orchid has yellow and purple flower colour morphs that are maintained as a result of negative-frequency dependent selection due to behavioural responses by pollinators to a lack of reward availability (Gigord *et al*. 2001). Rare-colour morphs continue to persist in the population as pollinators initially visit the most abundant colour morph, but due to the lack of nectar or pollen reward tend to then sample the rare colour morph in hopes of finding a nectar or pollen reward at this morph instead (Gigord *et al*., 2001). This puts the rare morph at a reproductive advantage as both the rewardless plant and the naiveté of the pollinators contribute to the maintenance of the rare morph in the population. This negative-frequency dependent selection results in the maintenance of flower colour polymorphisms within populations of *D. sambucina* as both the rare and abundant colour morph can continue to persist since one morph is not under selection. There are several examples of how negative-frequency dependent selection by pollinators maintains flower

colour variation in nature (e.g., Heinrich, 1975; Ackerman and Galarza-Perez, 1991; Smithson and Macnair, 1997).

B. Non-pollinator mediated selection

More recently, selection imposed by non-pollinator agents is suggested to also drive flower colour polymorphisms (Figure 1.1; Strauss and Whittall, 2006). While pollinators may discriminate against flowers based on their colour, there are examples where flower colour variation cannot be explained by pollinator-mediated selection (e.g., Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Carlson and Holsinger, 2015). Hence there is not enough evidence to prove that pollinators are the sole driver of the diversity in flower colour that we have today (Rausher, 2008). Non-pollinator mediated selection is an alternate hypothesis to pollinator-mediated selection and hypothesises that abiotic and other biotic (non-pollinator) factors could be acting as selection agents on flower colour. These biotic and abiotic agents have been found to directly and indirectly (through other pleiotropic traits) select for polymorphisms in flowers (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Irwin *et al.* 2003; Strauss and Whittall, 2006), fruits (Willson and O' Dowd, 1989; Whitney and Stanton, 2004; Whitney and Lister, 2004), and seeds (Traveset and Willson, 1998).

Flower colour polymorphisms may be maintained by differential tolerance to abiotic conditions, as a result of environmental heterogeneity and stress tolerance (e.g., Warren and Mackenzie, 2001; Dick *et al*. 2011). Flavonoids are produced in response to ultraviolet radiation, temperature, drought stress, and herbivore pressures (Green and Durnford, 1996; Schemske and Bierzychudek, 2001; Coberly and Rausher, 2003; Dick *et*

al. 2011; Arista *et al*. 2013). Unpigmented morphs (lacking anthocyanins) have been found to have a low tolerance to dry conditions and are fitter in well-watered conditions whereas pigmented individuals tolerate these conditions better and tend to have a higher reproductive output (Chalker-Scott, 1999; Warren and Mackenzie, 2001, Schemske and Bierzychudek, 2001; 2007; Strauss and Whittall, 2006). The production of anthocyanins is an indicator of the synthesis of other flavonol compounds, which allow plants to survive stressful conditions such as environments with low water availability (Goodwin, 1998; Gould *et al.* 1995). Anthocyanins are costly to produce and as a result are considered to have adaptive significance as they are only produced in conditions with low moisture availability (Warren and Mackenzie, 2001). Schemske and Bierzychudek (2001) showed that blue-flowered *Linanthus parryae* (A. Gray) Greene produced more seeds and had a fitness advantage in years of low spring precipitation, whereas white-flowered *L. parryae* were at a fitness advantage and produced more seeds in years of high spring precipitation. Another example of this is demonstrated in *Protea repens* (L.) L populations which have co-occurring pink and white morphs distributed along an elevational range with the frequency of pink morphs increasing with an increase in elevation (Carlson and Holsinger, 2015). This distribution of morphs occurs as a result of decreased temperature, and increased solar radiation at higher elevations (Carlson and Holsinger, 2015). Photoinhibition is caused by high levels of solar radiation and exacerbated by cold, drought, or nutrient stress (Steyn *et al*. 2002; Close and Beadle, 2003). Morphs containing anthocyanins have an advantage as they have increased tolerance to drought, cold, solar radiation, and nutrient deficiency thereby preventing photoinhibition from taking place (Coberly and Rausher, 2003; Dick *et al.* 2011;

Schemske and Bierzychudek, 2001). This is further illustrated by work on *Plantago lanceolata* L. (Stiles *et al*. 2007) and ornamental asters (Nissan-Levi *et al*. 2007) which both found that anthocyanin production increased in flowers and darker flowers were produced with a decrease in environmental temperature.

Both the type and location of anthocyanin pigments result in flower colour morphs having different tolerances to light, heat, and water availability (Daniel *et al.* 1999; Winkel-Shirley, 2002; Coberly and Rausher, 2003; Tattini *et al*. 2004; Tanaka *et al.* 2008; Agati and Tattini, 2010). Pigments such as cyanidin, delphinin and malvidin are high-hydroxylated flavonoids and have a higher fitness and are well adapted to drought conditions and environments with excess light. On the other hand, low-hydroxylated flavonoids like pelargonidin are less effective in these environmental conditions (Daniel *et al*. 1999; Winkel-Shirley, 2002; Tattini *et al*. 2004; Tanaka *et al.* 2008; Agati and Tattini, 2010). In addition, the location of the anthocyanins in plant tissue plays an important role in environmental stress response (Coberly and Rausher, 2003). For example, in *Ipomoea purpurea* (L.) Roth there are two different kinds of white flower colour morphs, one that lacks anthocyanins in both its vegetative and floral tissue and the other type that contains anthocyanins in its vegetative tissue, but lacks anthocyanins in its floral tissue. The white morph with anthocyanins in its vegetative tissue occurs at high frequencies in nature and is more tolerant to heat stress than the other white morph, which has decreased flower production and lower fertilization success in high temperature environments (Coberly and Rausher, 2003).

In some cases, flower colour itself and the pigments associated with it are not the primary targets of selection (Arista *et al*. 2013). Since anthocyanins are not only

responsible for flower colour, but also many other physiological traits, it is plausible that one of these other traits (other than flower colour) is instead under selection. Thus other plant physiological traits can be important selection factors that indirectly affect flower colour (Levin and Brack, 1995; Armbruster *et al*.1997; Armbruster, 2002; Frey, 2004). A number of extrinsic factors, such as exposure to metals in the soil, the shape of epidermal cells, and the concentration of vacuolar sugars have been linked to flavonoid (including anthocyanin) production and changes in floral pigment (Miller, 2011).

C. Both non-pollinator and pollinator mediated selection

There have been several recorded instances where both non-pollinator and pollinator mediated selection act on flower colour variation and polymorphisms in combination. Two of the most notable, *Lysimachia arvensis* (L.) U. Manns & Anderb. and *Raphanus sativus* (L.), both have within population flower colour polymorphisms which are maintained by non-pollinator selective forces such as climatic factors and herbivory, countered by pollinator-mediated selection and reproductive strategy (Arista *et al*. 2013; Irwin *et al*. 2003; Irwin and Strauss, 2004; Ortiz *et al*. 2015). The proportion and fitness of blue *L. arvensis* morphs was found to increase in areas with hotter and drier Mediterranean climates whereas red morphs were favoured in wet and shaded temperate, oceanic habitats (Arista *et al.* 2013). In some Mediterranean populations of *L. arvensis,* many blue morphs frequently occur alongside a low proportion of red morphs, which continue to persist as a result of delayed selfing that occurs at the end of the season. This delayed selfing mechanism maintains the blue and red polymorphisms of *L. arvensis* in these Mediterranean populations as it diminishes the effect of both biotic and abiotic selective agents (Ortiz *et al.* 2015). *R. sativus* laboratory experiments suggested that

flower colour polymorphism in the species was driven by pollinator-mediated selection, which favoured the yellow morph over the pink, white, and bronze morphs (Irwin and Strauss, 2004). Studies of natural populations of *R. sativus* however, indicate that all four morph frequencies have remained relatively constant over subsequent generations as a result of a counter-selective herbivore pressure which was influenced by anthocyanin pigments in floral tissues (Panetsos, 1964; Irwin *et al*. 2003; Irwin and Strauss, 2004). *R. sativus* colour morphs have differences in plant defensive chemistry which results in white and yellow morphs being more attractive to certain herbivores than pink and bronze colour morphs (Irwin *et al*. 2003; Irwin and Strauss, 2004).

UNPIGMENTED MORPH		PIGMENTED MORPH
↑	Temperature	↓
↑	Water Availability	↓
↓	Herbivore Pressure	↑
↓	Solar Radiation	↑
↑	Soil Nutrients	↓

Figure 1.1. A diagramatic representation of the non-pollinator mediated selection agents that act on and select for pigmented and unpigmented flower colour morphs.

Rhodohypoxis baurii var. confecta

For the purpose of this study the flower colour polymorphisms within populations of *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard & Burtt was investigated. The Drakensberg near-endemic genus *Rhodohypoxis* Nel (Hypoxidaceae), commonly referred to as the red star, contains six species, which display magenta, pink, or white flowers. Unpublished data indicates the presence of anthocyanin pigments in the pink and magenta flowers (K.L. Glennon, unpublished data). *R. baurii* var. *confecta* is an ideal system to study the evolution of flower colour as this variety has naturally occurring colour morphs (magenta, pink and white) within the same population (Hilliard and Burtt, 1978). Hilliard and Burtt (1978) hypothesised that this variation was as a result of individual flowers changing colour throughout the flowering season (white to pink/magenta), which would mean that *R. baurii* var. *confecta* has developmentally transient pigment production and does not display true polymorphisms.

R. baurii var. *confecta* is a geophytic, perennial herb. Plants are small (<10cm in height) with linear, erect leaves that are covered in dense trichomes on the upper surface and margins (Figure 1.2). Individuals are capable of both sexual and asexual (vegetative) reproduction and are self-compatible (Biyela *et al*. unpublished data). They form scattered to dense populations of individuals that live in close proximity to one another. These populations occur at high altitudes in the Drakensberg alpine region at approximately 2800 m.a.s.l (Hilliard and Burtt, 1978, pers. obs.). Plants occur in habitats with shallow, rocky, and damp soils along the summits or plateaus of the escarpment. There are twelve known localities of *R. baurii* var. *confecta* across the Drakensberg Mountain Region (DMR; Hilliard and Burtt, 1978).

The three varieties of the *R. baurii* complex differ predominantly in flower colour and habitat. The other two varieties of the complex are *R. baurii* var. *baurii* and *R. baurii* var. *platypetala*, which are predominantly deep pink/magenta and bright white, respectively. Populations of *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* are usually monomorphic, however, *R. baurii* var. *platypetala* rarely have pale pink colour morphs within populations. The variable *R. baurii* var. *confecta* displays colours from both varieties (white, pink and magenta) in a single population. *R. baurii* var. *baurii* occurs in moist habitats in partly shaded locations whereas *R. baurii* var. *platypetala* occur in dry habitats with shallow, stony soils.

Aims and objectives

The overall aim of this study was to investigate the maintenance of flower colour using *Rhodohypoxis baurii* var. *confecta* as a study system. Findings from this study provided some understanding on the genetic mechanisms regulating flower colour in *Rhodohypoxis*. As no pollinator visitation has been observed to date, environmental variables that could potentially drive this trait variation across populations were examined. In order to fully understand how and why this variation in flower colour exists within *Rhodohypoxis baurii* var. *confecta* the study was divided into three main objectives (Figure 1.3):

1. To test whether *R. baurii* var. *confecta* represents a true polymorphisms or if individual flowers change colour over time as suggested by Hilliard and Burtt (1978)

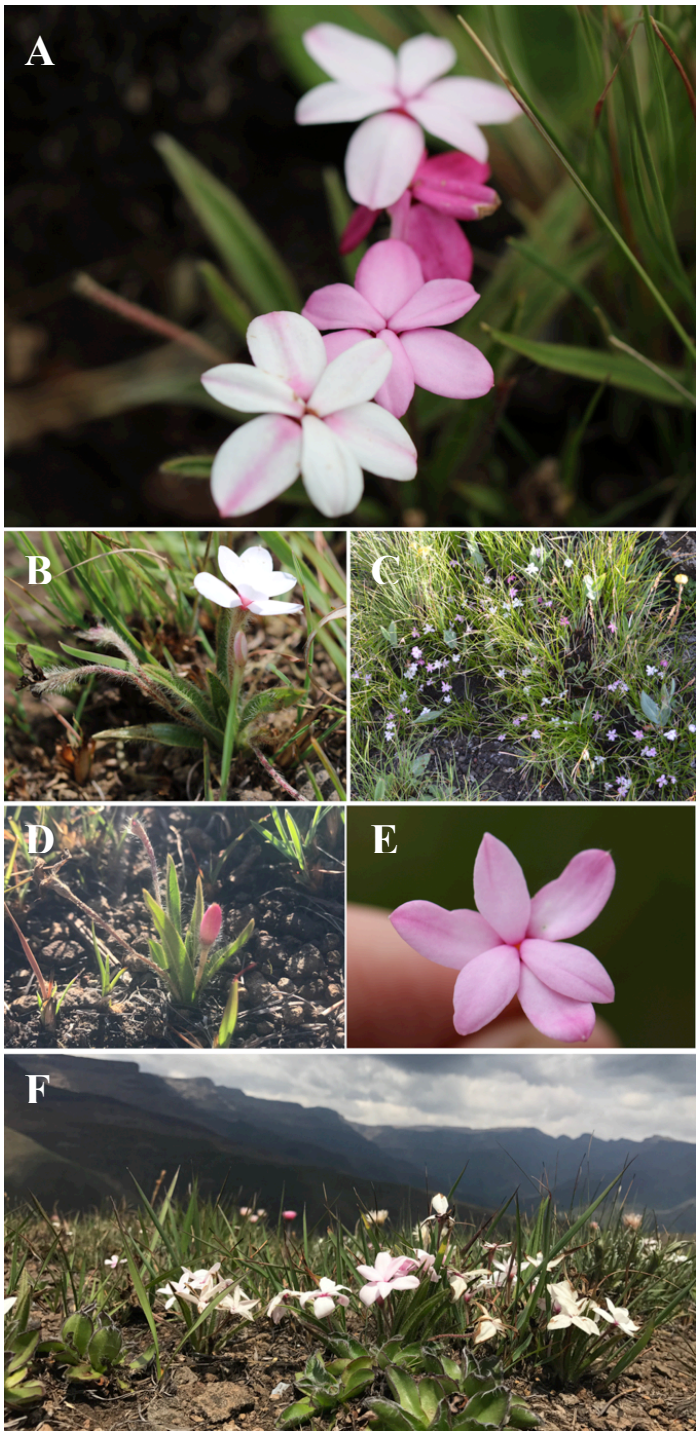

Figure 1.2. *Rhodohypoxis baurii* var. *confecta*. **A.** Range of colour morphs (magenta, pink, and white). **B.** Unpigmented morph with a bud and senescing flower. **C.** Mixed cluster of different coloured morphs. **D.** Pink bud and two senescing flowers **E.** Pigmented (pink) morph. **F.** Plants *in situ* at the Catchment population (EHS).

2. To test for a correlation between specific non-pollinator selection agents (temperature, precipitation, solar radiation, and soil moisture) and intra-population flower colour morphs within three *R. baurii* var. *confecta* populations
3. To test for the presence of expression in four anthocyanin biosynthetic pathway genes (*CHI, F3H*, *F3'H,* and *F3'5'H*) that effect flower colour morphs within a *R. baurii* var. *confecta* population

As this variation in flower colour contributes to the biodiversity of the Drakensberg region it is important to understand the mechanisms behind it so that biodiversity can be preserved. The genes responsible for flower colour are considered to be speciation genes as they result in pre-pollination prezygotic isolation (Rieseberg and Blackman, 2010). As a result, the presence of flower colour polymorphisms often leads to speciation (Bradshaw *et al*. 1995; van der Niet and Johnson, 2012). By investigating the nature of the observed flower colour variation in *R. baurii* var. *confecta* we can understand how this trait variation may contribute to potential diversification within a *Rhodohypoxis baurii* lineage. *Rhodohypoxis baurii* var. *confecta* is a near-endemic Drakensberg species and occurs in isolated, high altitude populations. As such, it is a prime system for the study of evolution, natural selection and maintenance of variation in populations.

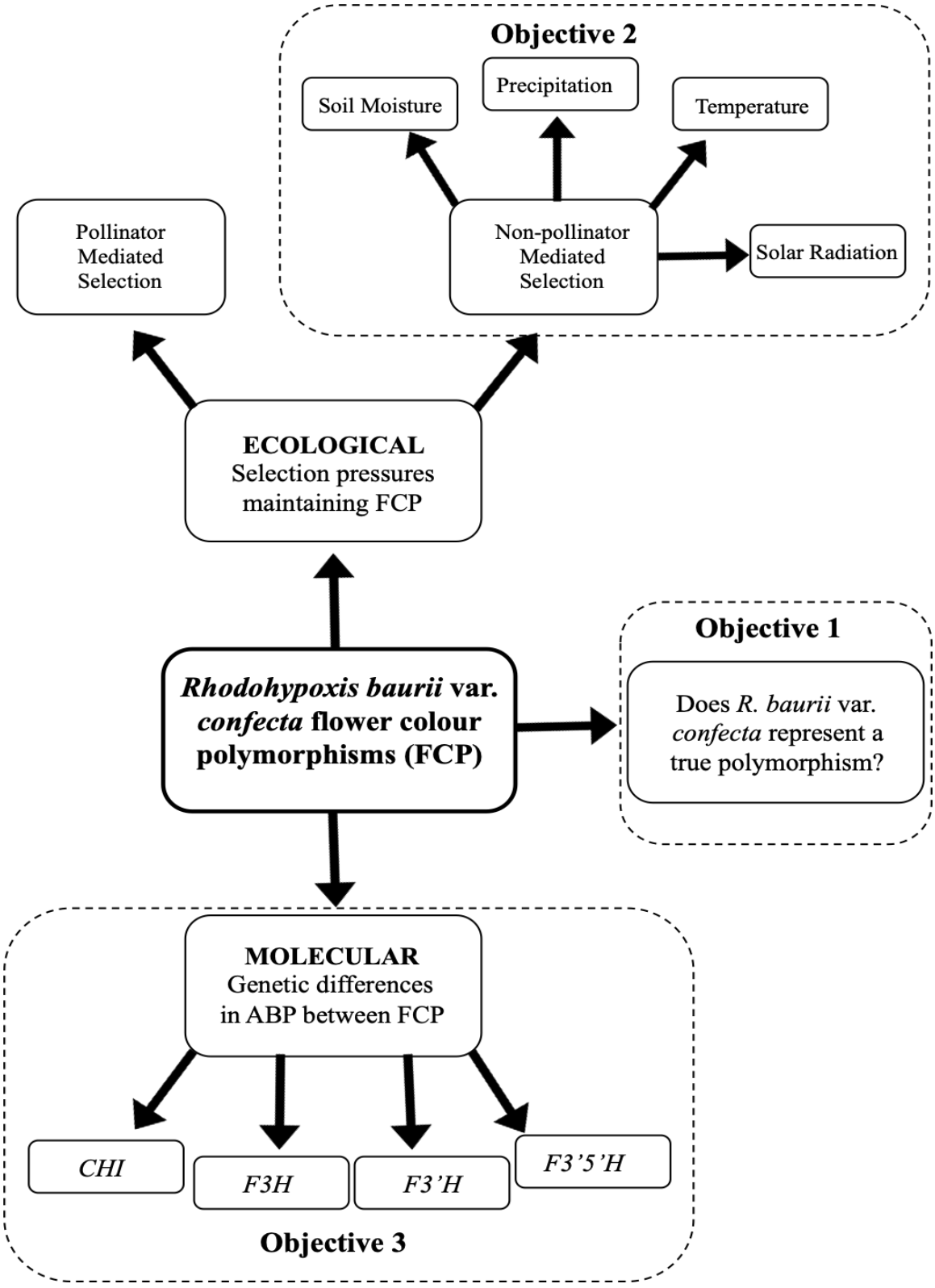

Figure 1.3. A diagrammatic representation of the objectives of this study.

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CHAPTER TWO

THE ROLE OF NON-POLLINATOR SELECTION AGENTS ON FLOWER COLOUR POLYMORPHISMS OF *RHODOHYPOXIS BAURII* VAR. *CONFECTA*

Abstract

Flower colour variation is both driven and maintained by either non-pollinator mediated selection agents, by pollinator selection agents or a combination thereof. Populations of *Rhodohypoxis baurii* (Baker) Nel. var. *confecta* Hilliard and Burtt comprise naturally occurring magenta, pink, and white flower colour morphs. Hilliard and Burtt (1978) hypothesised that this variety in flower colour can be attributed to individual *R. baurii* var. *confecta* flowers changing colour over a single flowering season (opening as white and changing to pink). In order to investigate whether *R. baurii* var. *confecta* flowers changed colour as suggested by Hilliard and Burtt (1978) or alternatively displayed true flower colour polymorphisms, individual flowers were tagged and monitored over the flowering season. In addition, the frequency of unpigmented (white) and pigmented (pink/magenta) morphs was measured between October and December to determine whether flower colour frequency changed over the flowering season. The extent to which non-pollinator selection agents correlated with this intra-population variation was then investigated by measuring the change in soil moisture, temperature, precipitation, and solar radiation over the flowering season (October- December). This study focused on three populations of *R. baurii* var. *confecta* located at Sentinel Peak near the Royal Natal National Park, South Africa. *R. baurii* var. *confecta* was found to display true flower

colour polymorphisms as individual flowers remained the same flower colour from opening until senescence. One population (SCP) shifted from a greater proportion of unpigmented morphs at the beginning of the flowering season to a relatively greater proportion of pigmented morphs at the end of the season whereas, the other two populations (EHS and WHL) maintained primarily unpigmented morphs throughout the season. At SCP a positive correlation was found between the proportion of pigmented morphs and soil moisture. This suggests that the accumulation of water in the soil promotes the production of pigmented flowers in *R. baurii* var. *confecta*. This is not supported by other studies, which have found that pigmented morphs are more tolerant and tend to have a higher fitness in environments with low moisture availability (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Strauss and Whittall, 2006). These results highlight the importance of non-pollinator mediated selection on the presence and maintenance of flower colour polymorphisms.

Key words: Drakensberg, environmental variables, Hypoxidaceae, Soil moisture, selection, trait variation

Introduction

Plant diversification and variation is shaped by selection. This is particularly evident in floral traits such as nectar reward, phenology, flower size, morphology, odour, and flower colour, all of which are the products of selection (Schiestl and Johnson, 2013). Variations in these traits are largely selected for and driven by pollinators. Variation in flower colour however, is driven by a variety of diverse selection forces that include both pollinator as well as non-pollinator selection agents (abiotic and biotic). As a result,

flower colour promotes divergent selection, which contributes to non-random mating and reproductive isolation (Dobzhansky, 1937; Kay, 2006; Sobel and Chen, 2014; Sobel and Streisfield, 2015). It is therefore considered the "magic trait in speciation" as it plays an extremely important role in driving speciation in plants (Funk *et al*. 2006; Nosil *et al.* 2009; Nosil, 2013; Sobel and Streisfield, 2015).

Flower colour variation is largely attributed to selective pressure exerted by pollinators (e.g. Fenster *et al.* 2004). Flower colour acts as a visual cue and stimulates the sensory system of pollinators (Schoonhoven *et al.* 2007; Kelber and Osorio, 2010). As a result, flower colour has a direct influence on pollinator attraction and acts as a selection target for visitors (Stebbins, 1974; Meléndez-Ackerman *et al.* 1997). The reproductive success, mating patterns, and fitness of plants are therefore ultimately determined by pollinator visitation (Brown and Clegg, 1984; Rausher *et al*. 1993; Levin and Brack, 1995; Dodd *et al.* 1999). Pollinators are attracted to a suite of specific plant traits known as pollinator syndromes (Fenster *et al.* 2004). Pollinator syndromes are made up a variety of plant traits including; flower colour, nectar, flower shape, flower size, sexual systems, and flowering phenology, which are selected for and vary depending on pollinator preference (Grant, 1949; Stebbins, 1974; Melendez-Ackerman and Campbell, 1998; Hodges *et al.* 2002; Schiestl and Johnson, 2013). Pollinators therefore show preference for particular flower colour morphs over others (Medel *et al.* 2003). For example, hummingbirds favour *Mimulus* L. flowers that contain a high anthocyanin content and a large nectar reward while bees prefer *Mimulus* flowers with a low anthocyanin and carotenoid content (Schemske and Bradshaw, 1999).

Historically, pollinators have been recognised as the primary driving force behind flower colour variation however, non-pollinator mediated selection has been found to also play a role in flower colour variation. The anthocyanin biosynthetic pathway, one of the major pathways responsible for flower colour, is pleiotropic. This pathway is linked to a number of plant physiological traits other than flower colour (Mol *et al.* 1993; Koes *et al*. 1994; Walker *et al*. 1999; Spelt *et al*. 2002; Winkel-Shirley, 2002; Morita *et al*. 2006). These traits are related to plant environmental stress response and fitness and as a result are also under selection. In some instances pollinators are not the strongest selecting force acting on flower colour and in fact other abiotic and biotic selective agents are acting on the other physiological traits associated with the pathway (Levin and Brack, 1995; Schemske and Bierzychudek, 2001, 2007; Warren and Mackenzie, 2001; Coberly and Rausher, 2003; Strauss and Whittall, 2006; Arista *et al*. 2013; Carlson and Holsinger, 2015; Veiga *et al*. 2016). As a result, environmental conditions play a role in maintaining flower colour variation as different flower colour morphs exhibit different tolerance to abiotic conditions (Schemske and Bierzychudek, 2001; Strauss and Whittall, 2006).

Anthocyanin production initially evolved in response to ultraviolet light, drought and other environmental stressors whereas pollinator attraction evolved secondarily (Rausher, 2006). Plants containing anthocyanins in both vegetative and floral tissues have a higher fitness, survivorship, and tend to tolerate low temperatures, low water availability, low nutrient availability, and high solar radiation better than plants that do not contain anthocyanins and have white (unpigmented) flowers (Levin and Brack, 1995; Grace and Logan, 2000; Warren and Mackenzie, 2001; Schemske and Bierzychudek, 2001, 2007; Steyn *et al*. 2002). Anthocyanin pigments are costly to produce and therefore

are only produced in certain environmental conditions when plants need to survive harsh conditions as other flavonol compounds which allow plants to survive these stressful conditions are also synthesised alongside anthocyanins (Goodwin, 1998; Gould *et al.* 1995; Warren and Mackenzie, 2001). In laboratory experiments, Warren and Mackenzie (2001) found that in five polymorphic species white morphs were less tolerant to dry conditions. Their finding was corroborated by a field study on *Linanthus parryae* (A. Gray) Greene where white morphs were favoured in years of high spring precipitation and blue flowers had a fitness advantage in years of low spring precipitation (Schemske and Bierzychudek, 2001). White flower colour morphs that lack anthocyanins often do not have anthocyanins in their vegetative tissue either, which is why these morphs tend to be less adapted to stressful environmental conditions. This is best demonstrated in a study conducted on *Ipomoea pupurea* (L.) Roth where two different kinds of white flower colour morphs exist. One type of morph lacks anthocyanin pigments in both its vegetative and floral tissue whilst the other type of white morph contains anthocyanins in its vegetative tissue but lacks pigments in its floral tissue as a result of a mutation in a tissue-specific regulatory gene (Coberly and Rausher, 2003). The white morph that contains anthocyanins in its vegetative tissue occurs at high frequencies in nature and is more tolerant to heat stress than the white morph that does not contain anthocyanins in its vegetative tissue. In addition, the white morph that lacks pigment in both vegetative and floral tissue has decreased flower production and lower fertilization success in high temperature environments than pigmented individuals (Coberly and Rausher, 2003).

A combination of both pollinator and non-pollinator mediated selection can be responsible for shaping flower colour variation. These two types of selection can work

together through reinforcing selection or work against one another antagonistically (Strauss and Whittall, 2006). In addition, both forms of selection can contribute towards speciation through divergent and reinforcing selection. In terms of pollinator-mediated selection, pollinators favour and visit one colour morph over another, which thereby reduces gene flow between different colour morphs (Schemske and Bradshaw, 1999; Hodges *et al.* 2002; Bradshaw and Schemske, 2003). This would ultimately result in reproductive isolation, which would eventually drive divergence between different flower colour morphs (Servedio *et al.* 2011). In the context of non-pollinator mediated selection, flower colour morphs differ in terms of their tolerance to different environmental conditions. Different flower colour morphs would then occur in different habitats or at different times of the year according to their environmental stress tolerance and become reproductively isolated and eventually diverge (De Meeûs *et al.* 1993; Dick *et al.* 2011; Carlson and Holsinger, 2015).

It is well established that flower colour polymorphisms are determined by a number of selective agents including either, or a combination of non-pollinator and pollinator selection agents. Species are considered to be polymorphic if two or more discrete morphs coexist within one population. A polymorphism is defined as a discrete variant at a high frequency within a population that is not the result of recurrent mutations (Ford, 1940; Huxley, 1955; Narbona *et al.* 2018). Flowers that individually change colour during its lifespan is not considered a polymorphism (Narbona *et al.* 2018). In addition, species may have polymorphic and monomorphic populations (Wright, 1978; McLean and Stuart-Fox, 2014; Forsman, 2016).

Rhodohypxis baurii var. *confecta* has populations containing naturally occurring white, pink, and magenta flower colour morphs (Figure 2.1). Hilliard and Burtt (1978) postulated that this variability in *R. baurii* var. *confecta* populations occurs as a result of individual flowers opening as white and then changing to pink and then magenta over the course of a flowering season. In this study, the presence and maintenance of flower colour polymorphisms in *R. baurii* var. *confecta* was investigated. Firstly, it was predicted that the colour morphs of *R. baurii* var. *confecta* represent true polymorphisms and that individual flowers do not change colour as suggested by Hilliard and Burtt (1978). This was tested through monitoring individual flowers throughout the flowering season in order to determine whether individual flowers changed colour over a single flowering season. Secondly, it was predicted that the frequency of these colour polymorphisms would shift in populations of *R. baurii* var. *confecta* over the course of a flowering season. This was measured by conducting colour frequency surveys and using these counts to calculate the proportion of pigmented (pink/magenta) and unpigmented (white) flower colour morphs at each time point throughout the flowering season. No pollinator visitation was observed at *R. baurii* var. *confecta* populations (unpublished data). As a result, non-pollinator mediated selection agents such as soil moisture, temperature, precipitation, solar radiation, or a combination thereof were investigated as potential drivers of colour variation in *R. baurii* var. *confecta*. This was done by measuring soil moisture, precipitation, temperature, and solar radiation and testing if these variables correspond with changes in flower colour morph frequency.



Figure 2.1 Colour morphs of *Rhodohypoxis baurii* var. *confecta.* **A.** Range of flower colour morphs (magenta, pink, and white). **B and C**. Colour morphs *in situ* in close proximity to each other,

Methods

Study species

The Drakensberg near-endemic genus *Rhodohypoxis* Nel. (Hypoxidaceae) consists of six species. This study focused on the variety *Rhodohypoxis baurii* var. *confecta.* This variety has naturally occurring colour morphs (magenta, pink, and white) within the same population (Hilliard and Burtt, 1978). *R. baurii* var. *confecta* forms scattered to dense populations of individuals that live in close proximity to one another. Individual plants produce between two and five flowers in a flowering season, which are staggered temporarily. They are found in shallow, rocky, and damp soils along the summits or plateaus of the escarpment and generally occur at high altitudes in the alpine region of the Drakensberg main escarpment (2500 m.a.s.l and above) (Hilliard and Burtt, 1978, pers. obs.). There are a total of twelve known populations of *R. baurii* var. *confecta*, which are widespread across the Drakensberg Mountain Region (DMR). These populations are found in Swaziland (two populations), the northern border of KwaZulu-Natal (two populations), the border between KwaZulu-Natal and Lesotho (three populations), in the southern part of the Eastern Cape (two populations) and in the eastern part of the Free State (two populations) (Hilliard and Burtt, 1978; Figure 2.2). The other two *R. baurii* varieties are *R. baurii* var. *baurii* (Baker) Nel and *R. baurii* var. *platypetala* (Baker) Nel and are predominantly deep pink/red and bright white, respectively. The variable *R. baurii* var. *confecta* appears to display colours from both *R baurii* var. *baurii* and *R. baurii* var. *platypetala* varieties as it has populations consisting of magenta, pink, and white flowers.

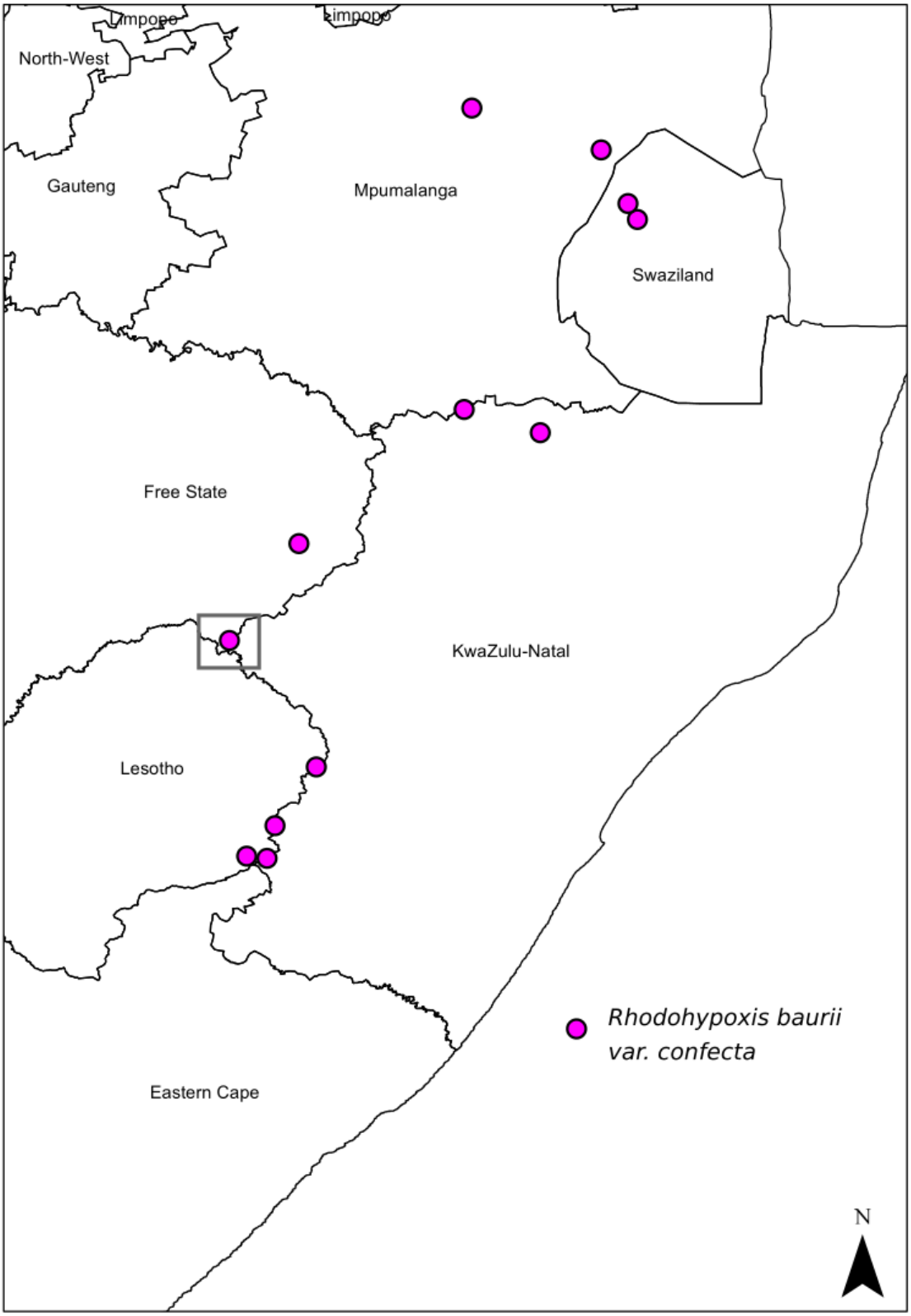

Figure 2.2 Known distribution of *Rhodohypoxis baurii* var. *confecta* localities in southern Africa (according to Hilliard and Burtt, 1978). Dark grey box indicates the location where the study was conducted.

Study sites

For the purpose of this study, three populations of *R. baurii* var. *confecta* that occur on the edge of the Royal Natal National Park (RNNP) in the Free State, South Africa were studied (Figure 2.3). The populations experience summer rainfall and occur in a mountain grassland biome. They are located near the Sentinel Peak Car Park (SCP), on a catchment (EHS) and behind the Witsieshoek Mountain Lodge (WHL). All three populations are approximately 2 km apart (SCP to EHS, and WHL to EHS, respectively). The furthest distance is between the SCP and WHL populations, which are 4.62 km apart. The populations were visited nine times, approximately every one to two weeks, throughout the flowering season (September to December). Preliminary data collection that took place between November and January 2016 suggested that a shift in the frequency of different flower colours occurs over the flowering season. A greater occurrence of white colour morphs relative to pink/magenta colour morphs were found in the early part of the flowering season. The number of white morphs decreased as the season progressed while the number of pink/magenta morphs increased.

The Sentinel Car Park population (SCP; 28°43’39”S, 28°53’29”E) occurs at the highest altitude (2550 m.a.s.l) near the parking lot for the Sentinel Peak trail. This population grows in high densities and is located on a west/northwest facing 45° mountain slope and the plants are widespread both above and below the Sentinel Peak hiking trail. Plants are exposed to sunlight from midday to early afternoon and experience some shading from the surrounding mountains in the morning. As a result of its positioning on the steep slope, the population experiences considerable water runoff after rainfall events.

The Catchment population (EHS; 28°42’14”S, 28°53’37”E) occurs on a flat catchment that can be accessed from the road leading to the Sentinel car park. It is at a lower altitude than the SCP population, but is higher than the WHL population (2249 m.a.s.l). Plants experience full sun throughout the day and are exposed to wind and direct rainfall, rather than runoff. *R. baurii* var. *confecta* individuals are less densely distributed than the SCP population and are widespread along the southwest side of the catchment. Most of the individuals are found in thin soil as a result of the underlying bedrock.

The Witsieshoek Lodge population (WHL; 28°41’11”S, 28°53’57”E) is located behind the Witsieshoek Lodge at the lowest altitude of the three populations (2195 m.a.s.l). The population has a similar habitat to the catchment population (EHS) in that it is mostly flat, has thin soil and is exposed to full sunlight throughout the day. The plants at WHL face south and at certain points in the population are located on a gradual slope.

Experimental design and protocol

In-situ flower colour frequency surveys of the three focal populations were conducted throughout the flowering season (October to December). In addition, specific individual plants within these populations were tagged and their colour was monitored. Colour frequency surveys were combined with measurements of environmental variables including temperature, soil moisture, solar radiation, and humidity in order to determine if there is a correlation between flower colour frequency and the change in these environmental variables over the flowering season.

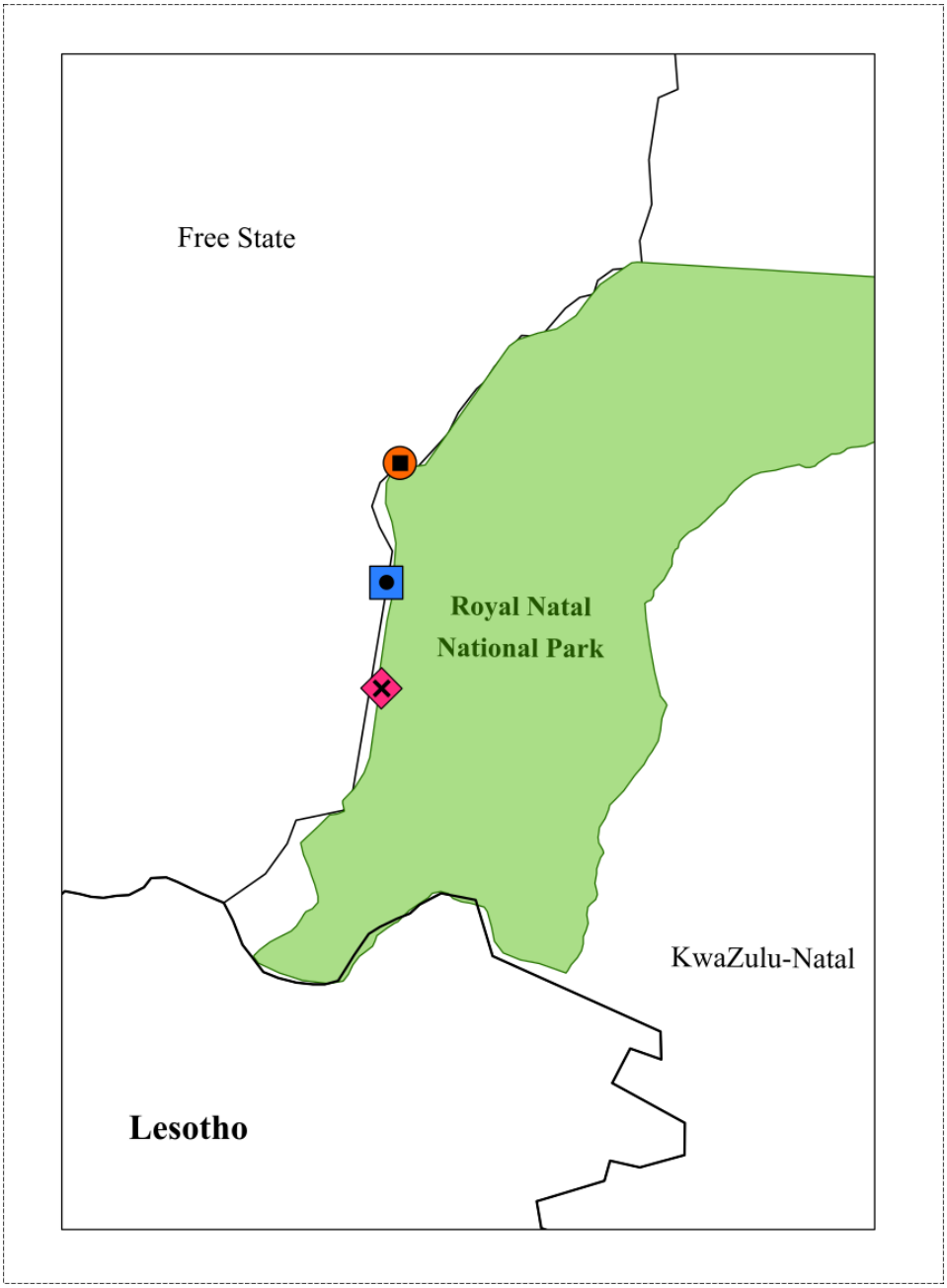

Figure 2.3 The location of the three study sites in the Sentinel Park area of the Royal Natal National Park**,** South Africa. The Sentinel Car Park (SCP) population is indicated by the pink diamond, the Catchment (EHS) population by the blue square, and the Witsieshoek Lodge (WHL) population by the orange circle.

Field counts of flower colours

Trips to Sentinel Peak were conducted in September, October, November, and December to cover the beginning, middle and end of the flowering season. This timeframe enabled us to establish the timing and pattern of flower colour transitions

within the three populations of *R. baurii* var. *confecta*. The trips were commenced on 19 September 2017 and semi-permanent transects were set up once flowers first emerged on 10 October 2017. By 29 December 2017, no new flowers had emerged and populations started to senesce. As a result seven trips where colour frequency counts could take place occurred between 10 October and 29 December 2017.

During each trip, a survey of the number of individuals for each colour (pink/magenta or white) was conducted at each population to test whether there was a change in the frequency of pigmented and unpigmented flowers over the flowering season. Longer trips, consisting of three to four days, were conducted during the middle of the flowering season when *R. baurii* var. *confecta* populations were most dense (November and early December). For these longer trips, daily counts were recorded and combined to calculate the average frequency of pigmented and unpigmented flowers for that trip. Colour surveys were conducted along three consecutive fixed transects in each of the three populations of *R. baurii* var. *confecta* (total of 9 transects). In order to cover most of the narrow and elongated population distribution, the three fixed transects at each population were spaced two meters apart and followed on consecutively from one another (Figure 2.4B). Each transect was one meter wide, downslope, and extended 50m in length. The frequency of white and pink/magenta flowers (unpigmented and pigmented, respectively) was estimated within the transect area using two clicker counters (Tally Counter, Eurohike, United Kingdom) (one counter = white/unpigmented, one counter = colour/pigmented)(Figure 2.4A). From these counts, the proportion of pink/magenta flowers in the population was calculated by dividing the number of pigmented flowers by the total number of flowers counted over one transect. The proportion of pigmented

colour morphs was used in further analyses as it showed how the number of pigmented morphs changed over the entire flowering season relative to the size of the entire population. The same transects were used for all field trips to monitor changes in the frequency of white to pink/magenta changes over the flowering season. When possible, surveys were conducted at the same time each day to account for the influence of available light on flower colour perception. Additionally, at the beginning of each frequency survey the visibility, cloud cover and weather was recorded, and temperature (°C), humidity (%), and wind speed (km/h) was logged with a Kestrel Pocket Weather Tracker 5500 (Kestrel Meters, PA, USA).

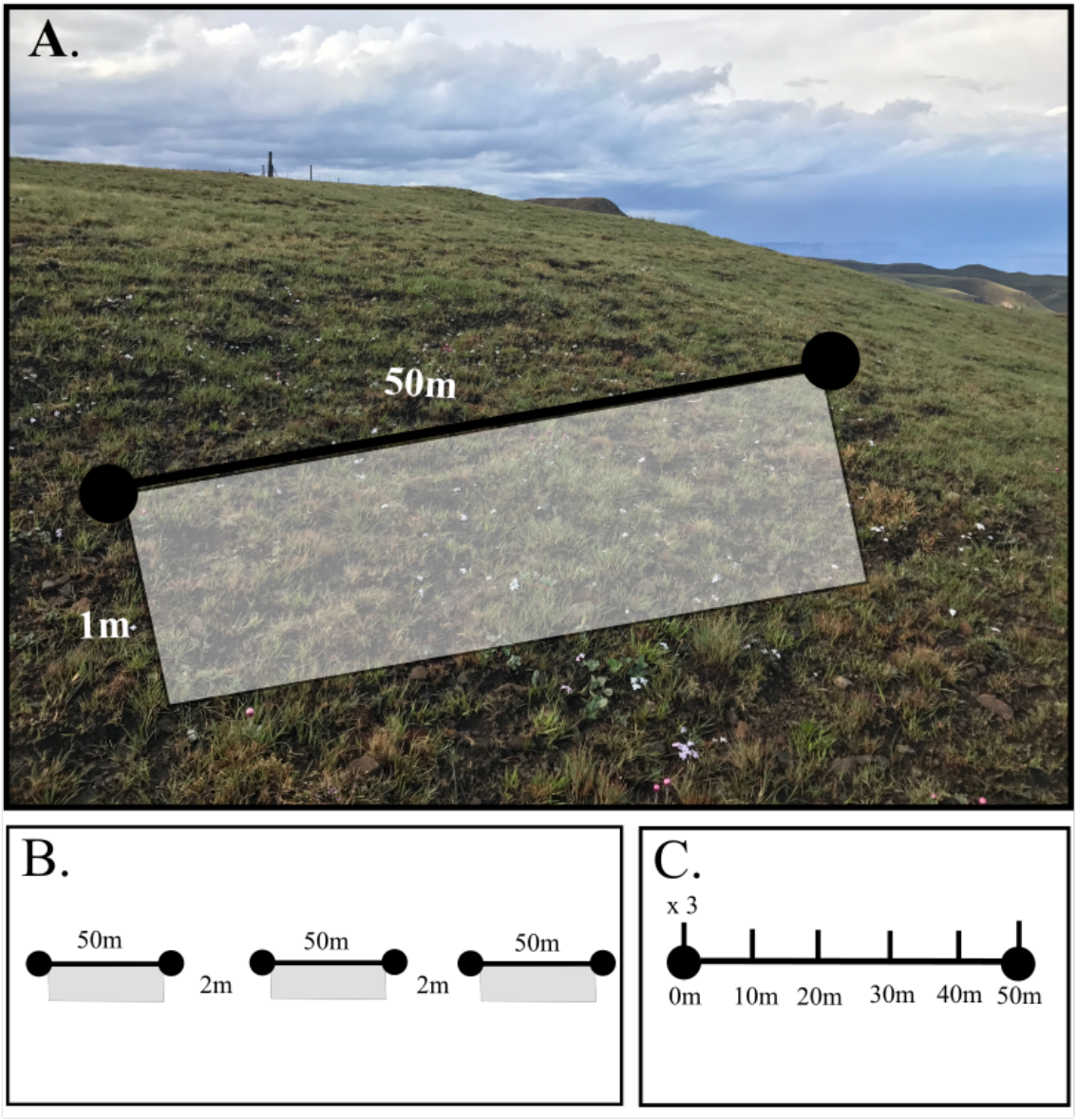

Figure 2.4. Representation of the semi-permanent transects used to measure flower colour frequency, and soil moisture. **A.** The layout of a transect *in situ* showing the area in which flower colour frequencies were measured. **B.** The positioning of the three transects set up in each population. **C.** The intervals at which soil moisture was measured. Three repeated measurements were taken at six points along each transect.

A non-parametric Friedman test was performed using the base *stats* package in RStudio version 1.1.456 (RStudio team, 2018) to test for a significant change in the proportion of pigmented flowers at each transect over the flowering season and between populations. A Nemenyi post-hoc test was then preformed to further interpret the Friedman test findings using the base *stats* package in RStudio version 1.1.456 (RStudio team, 2018).

Monitoring tagged plants and flowers

In November, during the height of the flowering season, a five plants along each of the three transects at SCP were tagged with silver metal tags every ten meters within each transect (total n = 15 plants). The flowers last for approximately 12-16 days; two to three days from bud to opening and then 14 days until flowers senesce. Individuals that had recently opened were selected and comprised flowers that were entirely white, entirely pink, or entirely magenta. In addition, more ambiguous morphs (e.g., white flowers with petals that had pink stripes or white flowers with pink perigone tubes) were included to test whether these flowers would become more pigmented over time (one or two individuals per transect, if present). Plants were tagged to monitor whether pigmented, unpigmented and ambiguously pigmented flowers changed colour over the flowering season or if they remained the same. In addition, tagging individual plants provided information on whether these plants were producing specifically white, pink, or magenta flowers as the season progressed or if the same plant produced different coloured flowers over the flowering season. This step was conducted in the middle of the flowering season (November) when pigmented and unpigmented morphs were being produced at an approximately equal rate. This ensured that there was a large enough

sample size to highlight any change in individual flower colour over its lifespan. Tagging was done when populations were visited for more than one day and trips were frequent. Detailed flower colour records and photographs were taken during each field trip to determine the pigmentation patterns of individual plants.

Abiotic agents of selection on colour polymorphisms

Preliminary fieldwork at the Sentinel Peak population suggested that pollinators were not a prime selection agent for the flower colour variation observed in *R. baurii* var. *confecta* populations. Therefore, non-pollinator selection agents (precipitation, temperature, solar radiation, and soil moisture) were studied as potential environmental drivers of this variation. The shift in the proportion of white to pink and magenta flowers is hypothesized to coincide with changes in solar radiation, rainfall and temperature over the flowering season. As *R. baurii* var. *confecta* species are generally found in damp environments, shifts in available water could also be an environmental cue for plants to shift flower colour.

At the beginning of the flowering season in September 2017, an iButton data logger (Maxim iButton DS1923 Fairbridge Technologies, South Africa) was placed at each of the three *R. baurii* var. *confecta* populations to record temperature throughout the flowering season. At WHL and EHS, iButtons were placed at the 12.5 meter point of the second (middle) transect. The iButton at SCP had to be well hidden due to tourist traffic on the trail and was placed 10m to the northwest of the beginning of the first transect. The iButtons at each population were approximately 10cm above the ground and were surrounded by the short grass found at each population. These iButtons were used to

measure the growing season temperatures from 10 September 2018 to 28 December 2018. At the end of the flowering season the iButtons were collected and the data were downloaded using OneWireViewer Version 1.5 (Maxim Integrated, CA, USA) and used to calculate average daily temperature for each population. Rain gauges could not be placed at each population due to tourism in the area, so a representative rain gauge was placed at the reception entrance of the Witsieshoek Mountain Lodge on the 21 September 2017 to estimate precipitation for the locality. An employee that is permanently based at the lodge recorded rainfall measurements daily at 08h30 throughout the flowering season. A seasonal Mann Kendall test was used to test if there was a significant difference in temperature and precipitation over the flowering season using the *Kendall* package (McLeod, 2011) in RStudio version 1.1.456 (RStudio team, 2018).

Soil moisture (volumetric soil water content) was measured during each visit using a soil moisture probe (CS658 HydroSense II Water Content Sensor, Campbell Scientific). Three measurements were recorded every ten meters along each 50m transect at the same time of day that the plants were counted (Figure 2.4C). A total of 18 soil moisture measurements for each transect were taken and the average, minimum, and maximum soil moisture for each transect was calculated to account for variability across a transect. For longer, three to four day trips, daily soil moisture measurements for each transect were taken and the average, minimum, and maximum soil moisture was calculated for each transect for the entire trip. As some points along the transects had thin soils, the soil moisture probe was not always fully immersed in the soil. If this occurred a reading would be taken and the depth would be noted. Weather conditions such as rainfall events before or during soil moisture readings were recorded during sampling. A

Friedman test was used to test for differences in soil moisture over the flowering season and between populations using the base *stats* package in RStudio version 1.1.456 (RStudio team, 2018). A Nemenyi post-hoc test was then preformed to further interpret these findings using the base *stats* package in RStudio version 1.1.456 (RStudio team, 2018).

Solar radiation measurements (kJ m^{-2} day^{-1}) over the flowering season (October – December 2017) were obtained from the WorldClim Version 2 database (Fick and Hijmans, 2017). Average solar radiation values for each month were downloaded using each population's GPS coordinates. This resulted in three solar radiation measurements for each population over the flowering season (October, November, December).

Generalized linear mixed models (GLMMs) were used to test if the proportions of pigmented flowers were associated with temperature, precipitation, solar radiation, soil moisture, and time within each population and transect using the *lme4*, *MuMin*, and *AICcmodavg* packages (Bates *et al*. 2015; Bartoń, 2017; Mazerolle, 2017) in RStudio Version 1.1.456 (RStudio team, 2018). Preliminary data analysis found that minimum and maximum soil moisture and temperature values were most informative of environmental stress and varied even more significantly over the flowering season when compared to mean temperature and soil moisture measurements. As a result, minimum soil moisture and minimum temperature values were selected for the GLMM. Minimum soil moisture, minimum daily temperature, solar radiation, and Julian date were set as predictor variables. Population was set as a random effect. Precipitation and soil moisture were highly correlated ($\rho=0.70$, $p=<0.001$) and therefore only soil moisture was used for the GLMM because precipitation has a direct influence on soil moisture and the two

measurements can be used interchangeably. All possible model combinations were examined for all three populations together. 15 possible model combinations were compared for each GLMM and the models were ranked according to their ΔAIC values. The model with the lowest ΔAIC value (< 4) was considered to best fit the colour data over the season. The results from the GLMM were used to determine whether there was a correlation between the percentage of pigmented flowers recorded at each transect over the flowering season and the measured abiotic selection agents (precipitation, solar radiation, temperature, and soil moisture). The GLMM was used to predict which, if any, environmental cues are driving the colour variations observed in the populations and to assess if there is a threshold value where environmental variables cue a shift in colour frequency. All plots were made using *ggplot* (Wickham, 2016) and associated packages in RStudio.

Results

Monitoring tagged plants and flowers

Tagged *R. baurii* var. *confecta* plants showed no change in flower colour from the time they were tagged until senescence. In all cases, individual flowers remained the same colour from when they opened until just before they began to turn brown. In addition, tagged plants produced the same colour flowers throughout the season. Plants that first produced unpigmented (white) flowers when tagged continued to produce white flowers, and plants that first produced pigmented (magenta/pink) flowers continued to produce magenta or pink flowers throughout the flowering season.

Field counts of flower colours

Results from a non-parametric Friedman test show that there is a significant difference among repeated measures of the percentage of pigmented flowers over time (χ^2=12.29, P=0.002). A Nemenyi post-hoc test indicated that there was a shift from a relatively greater percentage of white colour morphs early in the season (Oct) to a greater percentage of pigmented morphs by the end of the season (Dec) at the SCP population (Table 2.1; Figure 2.5). Conversely, no apparent difference was observed at the other two populations (WHL and EHS; Table 2.1; Figure 2.5).

Table 2.1. Nemenyi post-hoc test results from the Friedman test. These results show that there was a the difference between flower colour morph frequency (pink/magenta versus white) between some populations over the flowering season .

	SCP	**WHL**
WHL	0.002**	-
EHS	0.082	0.370

Note. Asterisks denote Significance values: ****P*<0.001, ** *P*<0.01, and * *P*<0.05

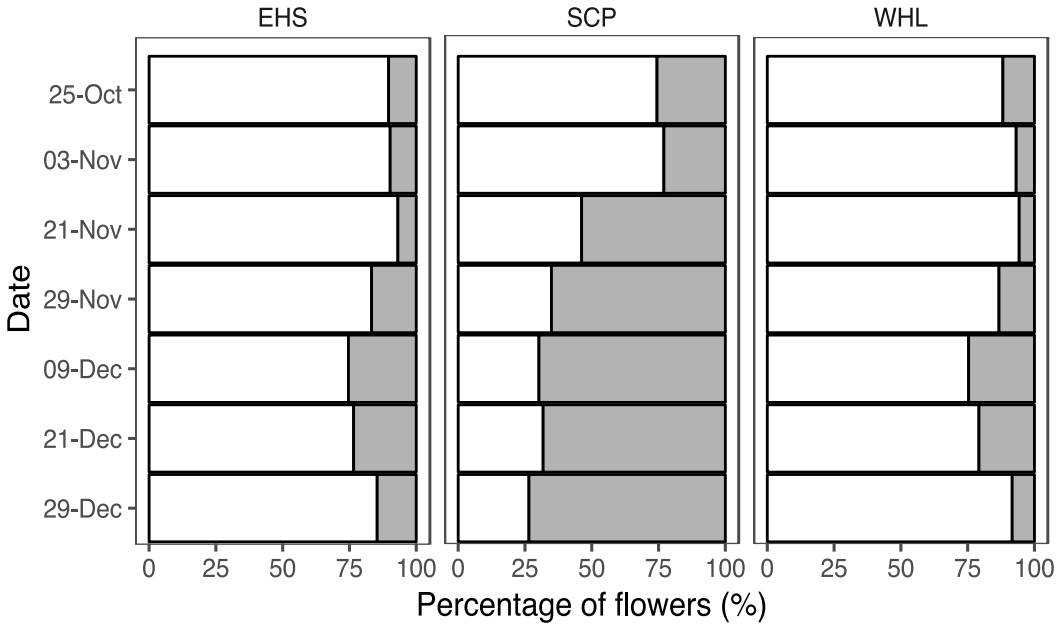

Figure 2.5. The relative percentages of pigmented (pink) and unpigmented (white) flowers measured at three populations (catchment [EHS], Sentinel Car Park [SCP], and Witsieshoek Lodge [WHL]) of *R. baurii* var. *confecta* over the flowering season. Grey and white bars represent the relative percentage of pigmented and unpigmented flower colour morphs, respectively.

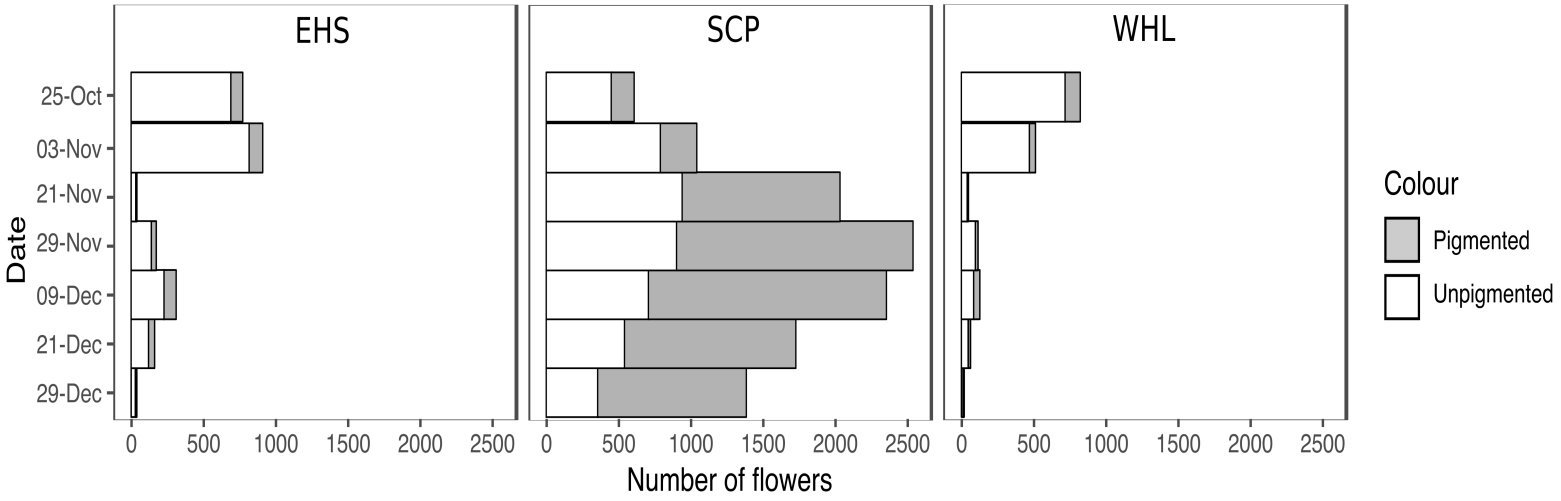

Figure 2.6. The actual counts of pigmented (pink) and unpigmented (white) flowers measured at three populations (catchment [EHS], Sentinel Car Park [SCP], and Witsieshoek Lodge [WHL]) of *R. baurii* var. *confecta* over the flowering season. Grey and white bars represent the relative percentage of pigmented and unpigmented flower colour morphs, respectively.

At SCP, there was a significant change in the percentage of pigmented and unpigmented flowers over the flowering season (Figures 2.5, 2.6, and 2.7). In the early parts of the flowering season (25 October) SCP comprised predominantly unpigmented flowers (>75%). In late November (21 November) the population had approximately equal percentages of unpigmented and pigmented flowers. By 29 November the percentage of pigmented flowers was greater than 60% in all three transects (Figure 2.7). The percentage of pigmented flowers continued to increase until it was greater than 70% in all three transects at the SCP population by the end of the flowering season (29 December).

There was no significant change in the percentage of pigmented and unpigmented flowers over the flowering season at the WHL and EHS populations (Figures 2.5, 2.6, and 2.7). At the WHL population the percentage of pigmented flowers slightly fluctuated throughout the season but remained lower than 30% of the population. On 9 December, the percentage of pigmented flowers was at its highest in transects two and three (< 28%; Figure 2.7). However, by the next sampling period the percentage of pigmented flowers had begun to decrease again in these two transects. By the end of the flowering season (29 December) the flowers in transect one had died back entirely and transects two and three contained a maximum of five flowers, where only one was pigmented. A similar trend was observed in the EHS population where the population had the highest percentage of pigmented flowers on 9 December (< 27%) and in subsequent surveys the percentage of pigmented flowers had decreased.

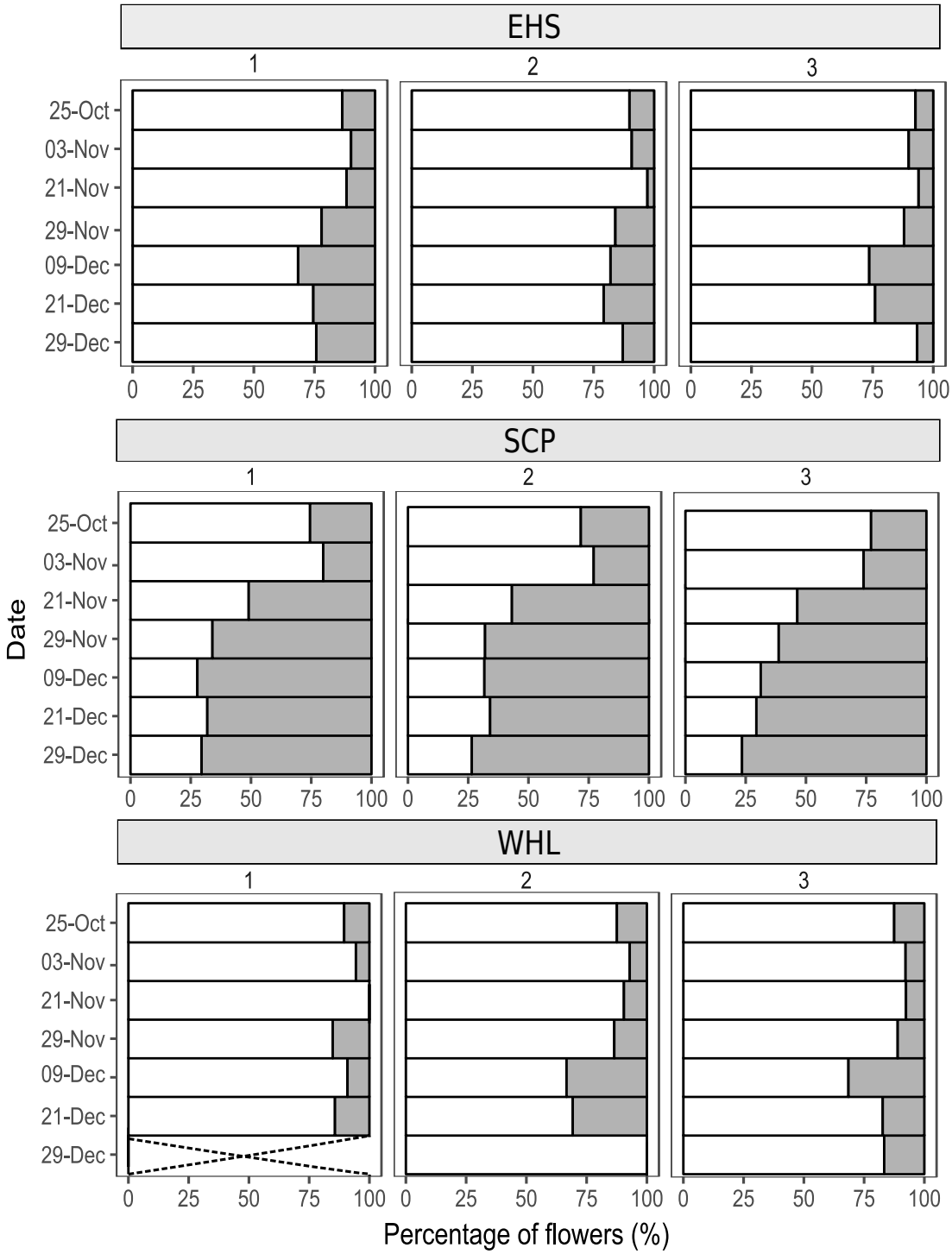

Figure 2.7. The average change in the relative percentages of pigmented (pink) and unpigmented (white) flowers measured at three permanent transects at the catchment population (EHS), Sentinel car park population (SCP), and Witsieshoek Lodge population (WHL) of *R. baurii* var. *confecta* over the flowering season. Grey and white bars represent the relative percentage of pigmented and unpigmented flower colour morphs respectively. Dashed lines represent absence of flowers.

Seasonal change in abiotic variables

There was a gradual increase in daily average temperature between early October and late December for both SCP (τ=0.22, *P*=0.001) and EHS (τ=0.25, *P*=0.0003; Figure 2.8). This same temperature trend was not observed at the WHL population (τ=0.04, *P*=0.56; Figure 2.8), which appeared to remain constant throughout the season. The monthly average solar radiation differed between each of the three populations. Overall, EHS experienced the highest solar radiation values followed by WHL and SCP, respectively.

There was a clear monotonic increase in the size and number of rainfall events in the Witsieshoek area over the summer season (τ = 0.24, *P*=0.002; Figure 2.9). Soil moisture at the SCP increased over the flowering season and after the middle of the season (29 November) was almost always above 30% soil moisture (χ^2=14, P=0.0009; Table 2.2; Figure 2.10). Over the entire flowering season, transects one, two, and three at SCP reached soil moisture maxima of 40%, 45%, and 44% respectively. Conversely, the WHL and EHS populations remained comparatively low with maximum values of 38%, 33%, and 27% (WHL transects one, two, and three) and 35%, 27%, and 28% (EHS Transects one, two, and three), respectively and only six individual soil moisture measurements recorded at various timepoints across the season surpassed 30% soil moisture at WHL and EHS.

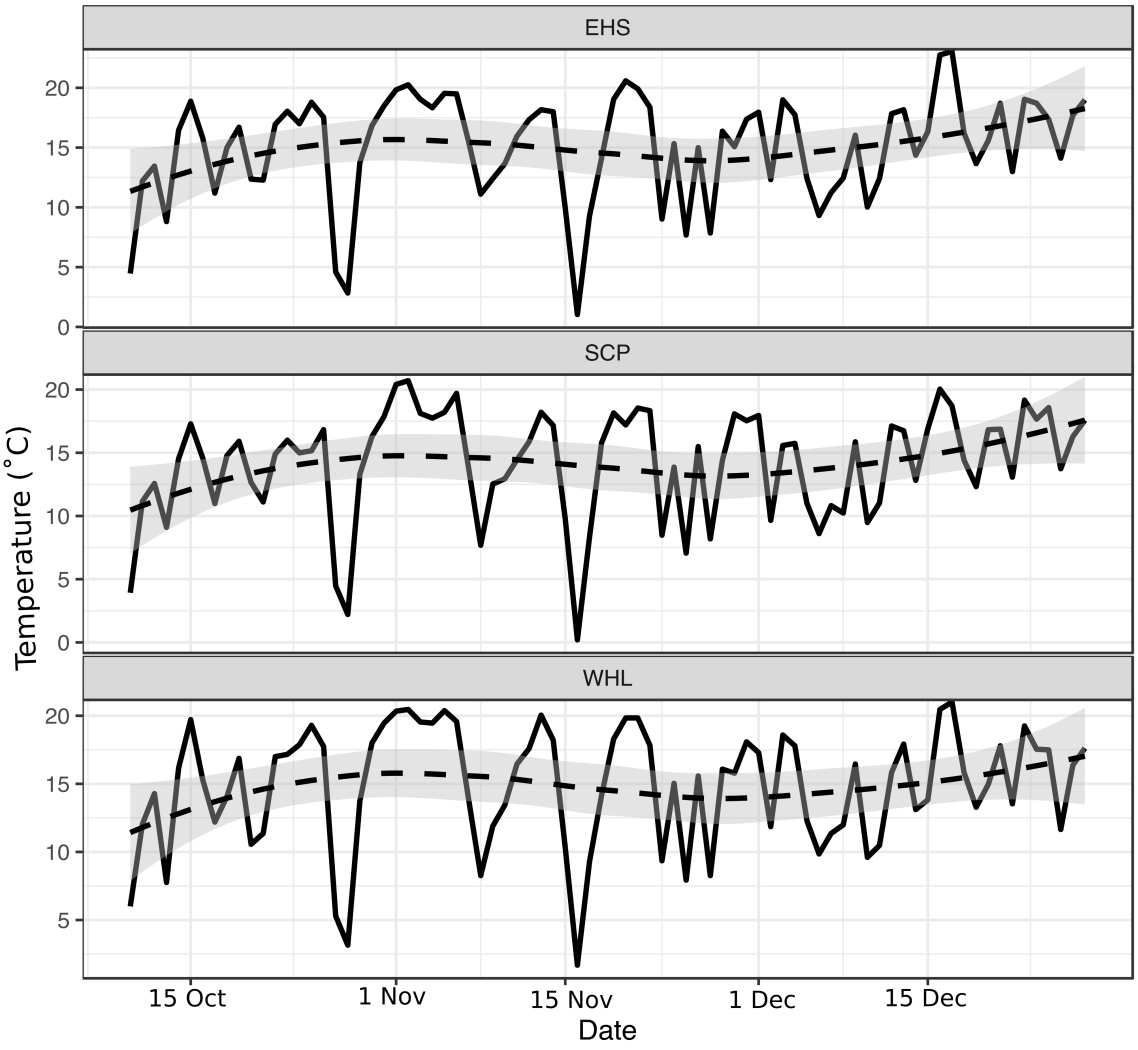

Figure 2.8. Daily average temperature over the 2017 flowering season (October–December) at the three populations of *R. baurii* var. *confecta*. Temperature was recorded at the catchment population (EHS), Sentinel car park population (SCP), and the Witsieshoek Lodge population (WHL).

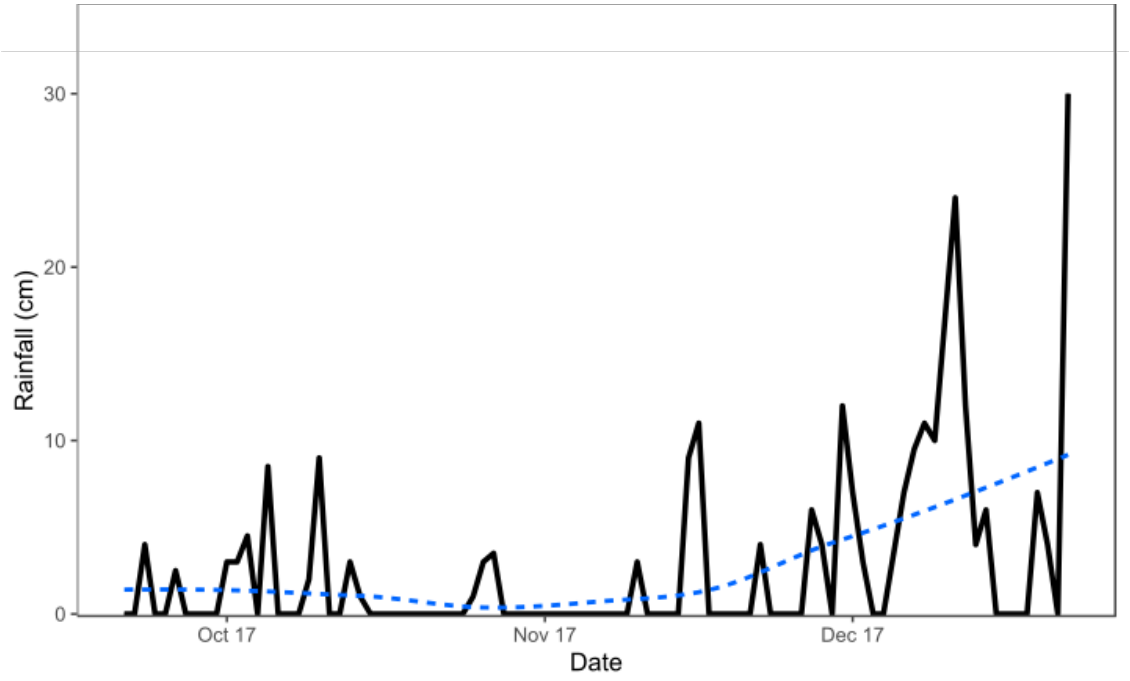

Figure 2.9. Daily rainfall (cm) in the Witsieshoek area from October to December 2017. Dashed line represents a positive monotonic trend over the season.

Table 2.2. Nemenyi post-hoc test results from the Friedman test that was used to test if soil moisture changed over the flowering season and between populations

	SCP	**WHL**
WHL	0.147	-
EHS	0.001	0.147

Note. Asterisks denote Significance values: ******P*<0.001, ** *P*<0.01, and * *P*<0.05

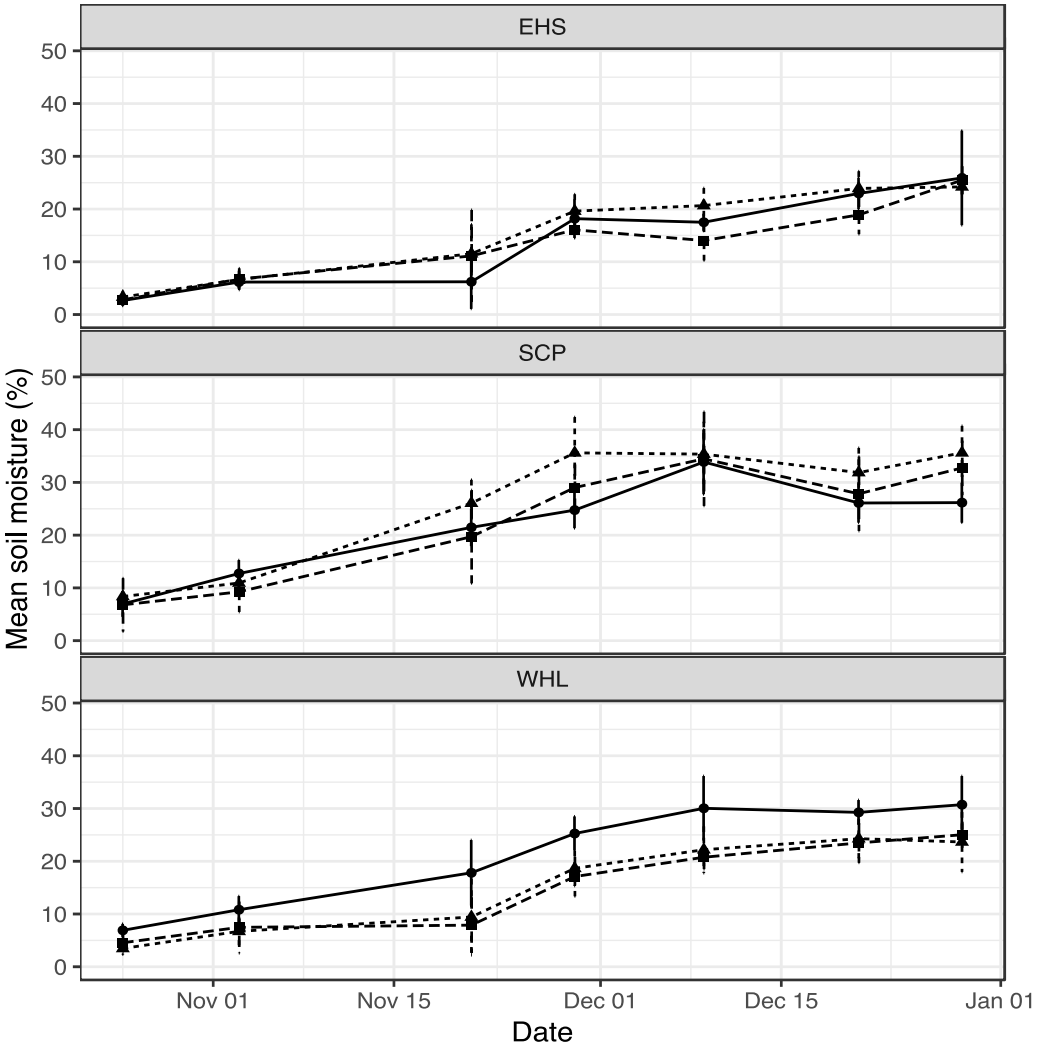

Figure 2.10. Average soil moisture at three transects within each of the three populations of *R. baurii* var. *confecta* over the flowering season (October to December). EHS is the catchment population, SCP is the Sentinel car park population, and WHL is the Witsieshoek Lodge population. Different line types (dotted, dashed, and solid) represent the three different transects measured at each population.

Potential relationship between colour frequency and environmental variables

The model that best fits the data (Table A.1- Appendix A) contained soil moisture and Julian date as predictor variables and population as a random effect. The results showed that both soil moisture and time had significant effects on the percentage of

pigmented flowers and that as these variables increase the percentage of pink flowers increase(Table 2.3). However, temperature was not a significant predictor (P=0.94). SCP had the greatest effect on the percentage of pigmented flowers (P<0.001).

Table 2.3. Generalized linear mixed model predictors and estimated effects to test which variables could be associated with an increase in the percentage of pink colour morphs across all three populations. This model includes soil moisture and Julian date with the estimated effects of these variables reported.

Note. Asterisks denote significance values: ******P*<0.001, ** ***P***<0.01, and * ***P***<0.05**

	Estimate	**Std. error**	**z value**	***P* value**
(Intercept)	3.09	0.33	9.41	<0.001***
Soil Moisture	0.14	0.03	4.73	<0.001***
Julian Date	0.21	0.03	7.24	<0.001***

A closer look at the data showed that there was a significant positive relationship between mean soil moisture and the percentage of pigmented flowers at SCP (r^2=0.62, P=0.0001; Figure 2.11) and at EHS (r^2=0.32, P= 0.0002; Figure 2.11 however, there is no significant relationship at the WHL population (r^2=0.06, P= 0.13; Figure 2.10).

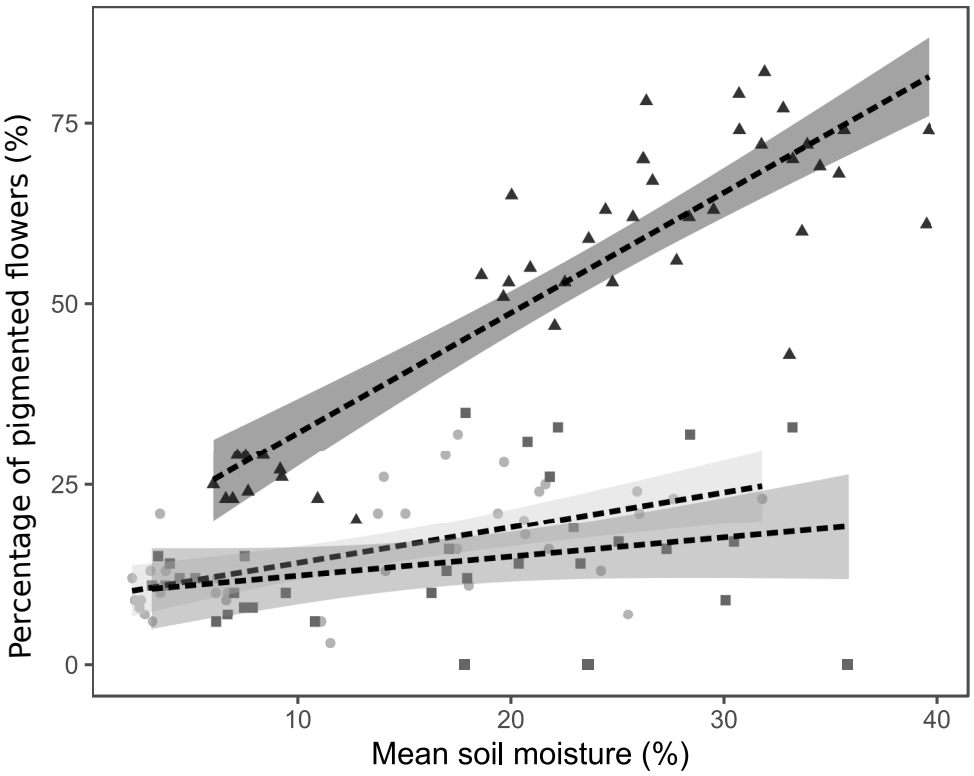

Figure 2.11: The relationship between mean soil moisture and the percentage of pigmented flowers at the three populations of *R. baurii* var. *confecta*. Triangles represent Sentinel Car Park population (SCP), circles represent the catchment population (EHS), and squares represent the Witsieshoek Lodge population (WHL).

Discussion

Rhodohypoxis baurii var. *confecta* populations display distinct polymorphisms of magenta, pink, and white. Instead of individual flowers opening as white flowers and gradually changing to pink and then magenta as described by Hilliard and Burtt (1978), *R. baurii* var. *confecta* morphs remain the same colour throughout their entire lifespan. Tagged plants that were monitored throughout the flowering season showed no apparent change in flower colour. Further, it did not appear that tagged plants put up flowers with different colour morphs and plants that first produced unpigmented (white) or pigmented

(pink/magenta) flowers continued to produce the same colour flowers over the course of the flowering season. In contrast, plants that do not display true polymorphisms and show flower colour change within individuals are usually triggered to change colour through the exposure to light (e.g., Willmer *et al*. 2009) or as a cue for pollinators (e.g., Weiss, 1991; Farzad *et al.* 2002). Therefore the presence of flower colour polymorphisms in populations of *R. baurii* var. *confecta* are most likely in response to other environmental factors.

The frequency of pigmented and unpigmented *R. baurii* var. *confecta* flowers shifted significantly over the flowering season in the SCP population. However, this same significant shift was not observed at the EHS and WHL populations (Figure 2.5). At the beginning of the flowering season (October) in the SCP, EHS, and WHL populations the percentage of unpigmented flowers was high relative to the percentage of pigmented flowers. In the SCP population as the season progressed the proportion of pigmented flowers increased steadily in the population until the end of the flowering season (December) when the population was made up of predominantly pigmented flowers. This same trend however, was not observed in the EHS and WHL populations where the proportion of pigmented flowers remained relatively low throughout the flowering season. Flower colour appears to be genetically controlled in individual plants and therefore this change in the proportion of polymorphisms over the flowering season is probably a result of local adaptation to varying environmental conditions (Schemske and Bierzychudek, 2001; Strauss and Whittall, 2006; Carlson and Holsinger, 2015; Forsman and Wennersten, 2016).

Temporal and spatial changes in environmental conditions are known to drive and maintain flower colour variation within populations (Schemske and Bierzychudek, 2001; Carlson and Holsinger, 2015; Narbona *et al*. 2018). Long-term studies have looked at shifts in flower colour polymorphisms over multiple flowering seasons (e.g. Schemske and Bierzychudek, 2001, 2007; Warren and Mackenzie, 2001; Arista *et al*. 2013; Veiga *et al*. 2016). However, to the best of my knowledge, there are no studies that analyse and have found changes in flower colour morph frequency within a single flowering season. Schemske and Bierzychudek (2001) conducted a well-known, long-term study that looks at changes in the population composition of flower colour polymorphisms in *Linanthus parryae*. The study was conducted over 11 flowering seasons and found that white *L. parryae* morphs are more abundant in years of high spring precipitation, whereas blue *L. larryae* morphs are favoured in years of low spring precipitation (Schemske and Bierzychudek, 2001). It is possible that fluctuations in flower colour morphs in the three populations of *R. baurii* var. *confecta* may exhibit a similar pattern, but additional, long-term seasonal data will be necessary to test this hypothesis.

In addition to temporal changes in environmental conditions, environmental heterogeneity on a spatial scale plays an important role in maintaining colour polymorphisms. The three populations of *R. baurii* var. *confecta* vary in their habitat and location. SCP is located at a much higher altitude (2550 m.a.s.l) compared to EHS and WHL which are found more than 200m lower at 2249 m.a.s.l and 2195 m.a.s.l respectively. In addition, SCP is located on a 45° slope, which experiences some shading throughout the day whereas EHS and WHL lie predominantly flat and are consistently exposed to sunlight throughout the day. The environmental conditions experienced at

each population are different and as a result, *R. baurii* var. *confecta* most probably adapts locally to these environmental conditions. *Protea repens* (L.) L is a good example of colour polymorphisms that are distributed over a heterogeneous environmental gradient, more specifically, an elevational cline (Carlson and Holsinger, 2015). *P. repens* populations are made up of pink and white morphs, and pink morphs are favoured at higher elevations compared to white morphs. This occurs because sites at higher elevation experience harsher environmental conditions and may experience lower temperatures, and higher ultraviolet radiation (Carlson and Holsinger, 2015). In this study, soil moisture, precipitation, solar radiation, and temperature were measured in each population over the flowering season in order to determine spatial and temporal differences in these environmental variables and to investigate whether they correlate with the frequencies of flower colour polymorphisms in *R. baurii* var. *confecta*.

Soil moisture appears to be related to the changes in flower colour that occurred in the SCP population. Soil moisture is closely correlated to the percentage of pigmented flowers in the population with the percentage of pigmented flowers increasing as soil moisture increased over the flowering season (Figure 2.10). The correlation between soil moisture and the percentage of pigmented flowers at the WHL and EHS populations is not as strong as the relationship found at the SCP population. In these two populations soil moisture increased gradually over the course of the flowering season, but remained relatively low when compared to the soil moisture at SCP. Towards the end of November, the soil moisture at SCP increased sharply to above 30% and consistently maintained levels of 30 to 45% soil moisture for the rest of the season. This large increase in soil moisture corresponded with the shift from a majority of unpigmented

plants in the population to a majority of pigmented plants in the population. Precipitation is highly correlated with soil moisture and has a direct influence on soil moisture. Increases in the proportion of pigmented morphs can therefore also be attributed to the increase in the amount and intensity of rainfall events that occur near the end of the flowering season.

The SCP population experiences greater runoff after rainfall events from adjacent areas as a result of the steep slope that the plants are located on, whereas EHS and WHL appear to experience little to no runoff, as these populations are located on relatively flat ground. In addition, these two populations tend to retain moisture in the soil for a much shorter period of time than SCP as a result of their constant exposure to sunlight throughout the day. The SCP population, on the other hand, experiences a relatively greater amount of time in the shade and as a result the soil in this area retains moisture for longer. There appears to be a critical level of soil moisture, which needs to be reached to facilitate the production of more pigmented flowers in the population. EHS and WHL have few instances where soil moisture reached levels higher than 30%, and in the last half of the flowering season soil moisture levels were not consistently maintained at levels above 30 to 45% as observed at the SCP population. The fact that the soil moisture at the WHL and EHS populations never reached the same level as it did at SCP could explain why there was no significant shift in the proportion of pigmented morphs in both of these two populations.

This finding is contradictory to other studies, which have found that pigmented flowers are favoured in environments with low moisture availability when compared to well-watered conditions (Schemske and Bierzychudek, 2001, 2007; Warren and

Mackenzie, 2001; Strauss and Whittall, 2006). Furthermore, unpigmented flower colour morphs have been found to have a lower tolerance to drought, cold, and UV radiation, whereas pigmented morphs are able to tolerate these environments (Schemske and Bierzychudek, 2001; Warren and Mackenzie 2001; Coberly and Rausher, 2003; Strauss and Whittall 2006; Rausher 2008; Dick *et al.* 2011). For instance, studies have found that unpigmented (white) flowers are less tolerant to dry conditions in both controlled experiments (Warren and Mackenzie, 2001) and in long-term field studies (Schemske and Bierzychudek, 2001) because anthocyanins are costly to produce in environments that are not under stress (Warren and Mackenzie, 2001). Warren and Mackenzie (2001) conducted drought experiments on five species with pigmented and unpigmented flower colour morphs and found that in all five species white morphs were less tolerant to conditions with low moisture availability. In some cases, unpigmented morphs contain anthocyanins in their vegetative tissue which help these plants tolerate stressful conditions better than unpigmented morphs without anthocyanins in both their floral and vegetative tissue (Coberly and Rausher, 2003). It is possible that whilst pigmented morphs that emerge at the end of the flowering season are not under any stress from low moisture availability, they may be under stress from water saturated soils. At the end of the flowering season, soil moisture levels reached a maximum of 45% and as a result, plants could experience water logging stress and anoxia. Morphs containing anthocyanins (pigmented morphs) could have a selective advantage in this stressful environment allowing plants to survive these conditions.

Another environmental variable that was measured was temperature which was found to change slightly over the flowering season however, this change was not as

closely correlated to the change in the proportion of pigmented flowers over the flowering season as soil moisture. Contrary to what was observed with soil moisture and despite the difference in altitude between the three populations, there was no noticeable difference between the temperatures measured at the different populations over the flowering season. Cold temperatures have been found to induce the production of anthocyanins in seedlings (Shichijo *et al.* 1993; Christie *et al*. 1994; Leyva *et al*. 1995; Graham, 1998), leaves (Krol *et al*. 1995; Oren-Shamir and Levi-Nissim, 1997) and twigs (Leng *et al.*1993). However, this does not coincide with what was observed in *R. baurii* var. *confecta* populations as few pigmented flowers were found in the colder early part of the season and the temperature gradually increased over the flowering season along with the production of pigmented flowers.

Another well-known cause of flower colour polymorphisms is solar radiation, which is known to induce anthocyanin production in plants (Chalker-Scott, 1999). The ABP responds to high levels of sunlight irradiance and produces compounds that protect plants against UV damage (Tattini *et al.* 2005; 2014; Rausher, 2008). Consequently, pigmented flowers could be emerging in response to increases in solar radiation and to protect the plants from UV damage. In this study, solar radiation was investigated as a possible environmental variable that may maintain flower colour polymorphisms in *R. baurii* var. *confecta*. There was a difference between solar radiation experienced at each of the three populations over the flowering season however, this variable did not contribute to the observed changes in flower colour.

Soil moisture was highly variable within each population of *R. baurii* var. *confecta* whereas other environmental variables such as temperature, and solar radiation

did not vary within a single population. It is therefore worthwhile to conduct future fieldwork to measure how soil moisture and the proportion of pigmented flowers change within a single population on a small scale. This can be addressed by measuring soil moisture and the number of pigmented flowers every one meter along the 25m transect. This work would help investigate whether spatially variable soil moisture has the same effect on the occurrence of pigmented flower colour morphs as temporal changes in soil moisture. These findings could further prove that soil moisture is responsible for maintaining flower colour polymorphisms in populations of *R. baurii* var. *confecta*.

R. baurii var. *confecta* has been described as displaying both white and pink/magenta morphs (Hilliard and Burtt, 1978). The SCP population displays these flower colour varieties and the frequency of these morphs change throughout the flowering season. The EHS and WHL populations however, had much fewer pigmented flowers that appeared to have a higher intensity and never displayed the dark magenta colours that were observed at SCP and described by Hilliard and Burtt (1978). These two populations appear to resemble another variety of the *R. baurii* complex, *R. baurii* var. *platypetala*, which has been described as having some instances where very pale pink flowers that occur in predominantly unpigmented/white populations (Hilliard and Burtt, 1978). The habitat description of *R. baurii* var. *platypetala* is similar to the EHS and WHL localities and the variety has been described as occurring in dry habitats with stony and shallow soil. In addition, the EHS and WHL localities are relatively drier in contrast to the damp grass slopes of the SCP locality. Further, the three populations are found at different altitudes and SCP is located at a much higher altitude than EHS and WHL respectively. According to Hilliard and Burtt (1978), *R. baurii* var. *confecta* occurs at

high altitudes, whereas *R. baurii* var. *platypetala* is more widespread and at lower altitudes compared to *R. baurii* var. *confecta*. On the distribution map of Hilliard and Burtt (1978) only *R. baurii* var. *confecta* occurs in the area in which the study was conducted and no instances of *R. baurii* var. *platypetala* have been recorded in the Sentinel Peak area. There is however a possibility that the EHS and WHL populations may be an unrecorded occurrence of *R. baurii var. platypetala*.

Alternatively, it is possible that *R. baurii* is a highly variable species that may respond differently to the same environmental variables. SCP could be a polymorphic population of *R. baurii* whereas EHS and WHL are relatively more monomorphic populations. The three varieties that make up the *R. baurii* complex are largely differentiated by flower colour and habitat. Monomorphic populations of white or magenta morphs (*R. baurii* var. *platypetala* and *R. baurii* var. *baurii*, respectively) could be a result of habitats with consistently low and high levels of soil moisture, respectively. This would closely align to the descriptions of *R. baurii* var. *platypetala* and *R. baurii* var. *baurii*, which were described as occurring in dry and moist habitats, respectively (Hilliard and Burtt, 1978). Polymorphic populations of *R. baurii* var. *confecta* would therefore be maintained by initially low levels of soil moisture, which would increase over the course of the flowering season. This would have to be tested for and further explored in future research.

Conclusion

Flower colour polymorphisms are not only maintained through pollinator-mediated selection, but can also be maintained by non-pollinator mediated selection including

environmental heterogeneity and stress tolerance (e.g., Warren and Mackenzie, 2001). *R. baurii* var. *confecta* displays white, pink and magenta flower colour polymorphisms within single populations. In this study, we show that the proportions of these polymorphisms change within a population during a single flowering season and that soil moisture may potentially prompt such a change. This is in contrast to other studies that have shown that pigmented flower colour morphs have a higher fitness and are more tolerant to environments with low moisture availability (Schemske and Bierzychudek, 2001; Mackenzie and Warren, 2001; Strauss and Whittall, 2006). Future work that explores how the variability of soil moisture at a finer scale influences the occurrence of pigmented flower colour morphs may provide further insight to non-pollinator mediated selection agents that promote or maintain these polymorphisms in a natural population.

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CHAPTER THREE

THE ROLE OF ABP GENES IN THE MAINTENANCE OF FLOWER COLOUR POLYMORPHISMS OF *RHODOHYPOXIS BAURII* VAR. *CONFECTA*

Abstract

Rhodohypoxis baurii (Baker) Nel. var. *confecta* Hilliard and Burtt has naturally occurring polymorphisms of pigmented (pink/magenta) and unpigmented (white) flower colour morphs. The anthocyanin biosynthetic pathway (ABP) is responsible for the production of pigment in *R. baurii* var. *confecta*. The ABP is pleiotropic and in addition to pigment production is responsible for traits related to plant physiology and environmental stress response. Consequently, the ABP is cited as being well conserved among angiosperm species (Quattrocchio et al. 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al.* 2014). The presence of expression in four ABP genes (*CHI*, *F3H, F3'H,* and *F3'5'H*) was tested for and compared between unpigmented and pigmented *R. baurii* var. *confecta* flowers. As there is no sequence data for *Rhodohypoxis* or any Hypoxidaceae species, primers for this molecular work were designed using closely related monocotyledon ABP gene sequences under the hypothesis that the gene sequences in the ABP are well conserved amongst angiosperms. Sequencing results showed that the amplified PCR products were not the targeted ABP genes. These results suggested that the designed primers were non-specific and that perhaps the gene sequences in the ABP were polymorphic and not as well conserved as literature suggests. The similarity of the four ABP genes within angiosperm species and more specifically within both monocotyledon and dicotyledon species was investigated. All available

complete sequences of the four regions of interest were aligned and sequence similarity was quantified. The results from this alignment indicate that the four ABP gene sequences are polymorphic and are most likely not well conserved within angiosperm species as a whole and, to some extent, within monocotyledon and dicotyledon species respectively. This suggests that the basic structure of the ABP is well conserved amongst angiosperm species however, the gene sequences that make up the pathway are polymorphic and less well conserved. The pathway is most likely polymorphic because the it is under a number of different selection pressures (non-pollinator and pollinator mediated selection), which vary between angiosperm species.

Keywords: Anthocyanins, conservation, monocotyledon, polymorphisms

Introduction

Flower colour is a highly variable trait amongst angiosperm species and is responsible for increasing phenotypic diversity. There are a wide variety of flower colours that vary in both hue (types of pigments) and intensity (concentrations of pigments). Flower colour variation results from the differential gene expression in the biochemical pathways responsible for the production of flower pigments. Differences in flower colour may result from varying patterns of gene expression, levels of expression, and in some cases the inactivation of some genes.

Although there is a vast array of flower colours, only three biochemical pathways are responsible for pigment production namely, betalains, carotenoids, and anthocyanins. These three biochemical pathways are generally considered highly conserved and have resulted in the independent evolution of similar flower colours across many flowering

plant lineages (Quattrocchio *et al*. 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al*. 2014). Betalains are limited to the order Caryophyllales and produce yellow-orange betaxanthins and red-violet betacyanins (Mabry, 1980; Steglich and Strack, 1990). Carotenoid pigments occur in both animals and plants and produce yellow, orange and red colouration (Goodwin, 1952). Anthocyanins are the most common of the three pigment classes and are widely distributed throughout flowering plant species (Clegg and Durbin, 2000). Anthocyanins give rise to a wide variety of colours ranging from pale yellow to blue to red (Tanaka *et al.* 2008).

The anthocyanin biosynthetic pathway (ABP) is responsible for the production of anthocyanins (Figure 3.1A and B). The molecular biology, colouration mechanism, biochemistry, and genetics of the anthocyanin biosynthetic pathway have been well described (Harborne, 1988; Stafford, 1990; Forkmann, 1991; Mol *et al*. 1993; Grotewold, 2006). Six consecutive enzymatic reactions result in pigment production and the seven associated genes that code for these enzymes have been isolated (Figures 3.1A and B) (Holton and Cornish, 1995; Mol *et al*. 1998). These genes are categorised into early biosynthetic genes (EBGs; *CHS*, *CHI* and *F3H*) and late biosynthetic genes (LBGs; *F3'H*, *F3'5'H*, *DFR*, *ANS* and *UF3GT*) (Liu *et al.* 2018). Variation in flower colour occurs through alterations in pigmentation intensity (concentration of pigment) and hue (types of pigments and co-pigments), which are dictated by the presence of expression and levels of expression of the ABP genes (Grotewold, 2006). There are three major types of anthocyanin pigments that are produced by the ABP: pelargonidin, cyanidin, and delphinidin (Holton and Cornish, 1995).

The presence of these three pigments is directly determined by *F3H, F3'H* and *F3'5'H* (flavone 3-hydroxylase, flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase; Figures 3.1A and B). *F3H* is responsible for the production of dihydrokaempferol (DHK), which ultimately leads to the production of pelargonidin-3-glucoside (otherwise known as anthocyanidins), a pigment that produces orange colours. The gene *F3'H* is responsible for the production of dihydroquercetin (DHQ), which produces cyanidin-3-glucoside and results in a reddish-purple. Lastly, *F3'5'H* is responsible for the production of dihydromyricetin (DHM), which goes on to produce delphinidin-3-glucoside responsible for blue hues. *F3'H* and *F3'5'H* both catalyse the hydroxylation of DHK to form DHQ (*F3'H*) and DHM (*F3'5'H*) and therefore expression of *F3H* is also required for the production of both cyanidin and delphinidin pigments (Tanaka *et al.* 2008). For example, the expression of both *F3'5'H* and *F3H* is required to produce the delphinidin pigment. Not all anthocyanin producing species produce all of the anthocyanin pigments. For example, snapdragons and maize do not produce delphinidin pigments and petunias do not produce pelargonidin pigments (Holton and Cornish, 1995). This is because in some instances, dihydroflavonol 4-reductase (*DFR*) cannot process some of the dihydroflavonols (DHK, DHQ, or DHM) produced as a result of strict substrate specificity.

The early biosynthetic genes (*CHS*, *CHI*, and *F3H*) are found at the beginning of the ABP and the expression of these genes is required for the production of other flavonoids including flavones, flavonones, and flavonols (Koes *et al.* 1994; Chalker-Scott, 1999; Grotewold, 2006; Ryan *et al*. 2002; Tanaka *et al.* 2008). Chalcone synthase (*CHS*) is the first gene in the pathway and provides the precursor for all classes of

flavonoids (Figures 3.1A and B; Grotewold, 2006). *CHS* is followed by chalcone isomerase (*CHI*), which is succeeded by *F3H*. These EBGs occur upstream of the LBGs, which are required for the production of pigments, but still play an important role in the production of pigment. For example, in potato tubers, the amount of expression of *CHS*, *CHI*, and *F3H*, was correlated to anthocyanin content as these genes were expressed at varying levels in both red and purple tubers and correlated to the corresponding polyphenol levels (André *et al*. 2009; Jung *et al*. 2009; Payyavula *et al.* 2013; Liu *et al*. 2015). In addition to the production of pigment, the expression of these genes is required for the production of a suite of other flavonoids that play an important role in plant physiology (Koes *et al.* 1994; Chalker-Scott, 1999; Grotewold, 2006; Ryan *et al*. 2002; Tanaka *et al.* 2008). Therefore, the ABP is directly related to plant fitness as it is linked to a number of plant physiological traits and can be considered pleiotropic. Not only is it responsible for determining flower colour, but it is also responsible for producing compounds that aid in plant disease defence, pollen viability, microbial interactions, trichome density, root hair formation, cell morphology, vacuolar size, and UV protection (Koes *et al*. 1994; Walker *et al*. 1999; Spelt *et al*. 2002; Morita *et al*. 2006).

Due to its pleiotropic nature, the pathway has been cited as being well conserved across angiosperm lineages (Quattrochio *et al.* 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al.* 2014). Flavonoids initially evolved to provide protection against UV radiation as plants moved onto land and the pathway has existed long before the evolution of flowering plants (van Tunen *et al.* 1988; Kubasek *et al.* 1992; Pelletier *et al.* 1997; Rausher, 2006). In flowering plants the modification of anthocyanidins is diverse and varies significantly between families

and species (Tanaka *et al*. 2008). As a result, homologous flower colours have evolved and originated independently in many different angiosperm lineages (e.g., Wilson *et al.* 2007).

Mutations are the major cause of phenotypic variation in the pathway and due to the nature of the anthocyanin pathway a single mutation can have profound effects on multiple phenotypic characters (Clegg and Durbin, 2000). Conversely, because of the pleiotropic effects of the genes in the pathway the extent at which mutations reach fixation and molecular decay occurs is limited (Coberly and Rausher, 2003; Streisfield and Rausher, 2011). Deactivation or reduced expression of any pathway enzymes or transcription factors in the ABP can result in loss of pigmentation (Grotewold, 2006). Anthocyanin pigment intensity is regulated by the MYB-bHLH-WD complex (MBW) . This complex is made up of proteins that control the regulation of the anthocyanin pathway genes (Broun, 2005; Koes *et al*. 2005; Ramsay and Glover, 2005), which belong to three gene families: the R2R3-MYB, basic helix-loop-helix (bHLH), and WD40-repeat (WDR) proteins. The R2R3-MYB proteins are responsible for specific anthocyanin regulation however, other copies of these proteins have become specialized to control specific traits such as trichome initiation and proanthocyanidin synthesis in seeds (Broun, 2005; Koes *et al*. 2005; Morita *et al*. 2006; Quattrocchio *et al*. 2006; Schwinn *et al*. 2006; Gonzalez *et al*. 2008; Sobel and Streisfield, 2013). Mutations that occur in R2R3-MYB, result in gains or losses in anthocyanin pigmentation and are responsible for differences in intensity and patterning of anthocyanin production in flowers (Cooley *et al*. 2011; Streisfield *et al*. 2013; Twyford *et al.* 2018). These gene mutations contribute to

phenotypic variation within species (Grant *et al*. 1998) and result in flower colour polymorphisms in populations.

Transitions from pigmented (e.g., magenta or pink) to unpigmented, (white) flowers may involve loss-of-function (LOF) mutations or *cis*-regulatory mutations to any pathway enzyme. Alternatively they may involve *cis*-regulatory mutations or LOF mutations to the regulator proteins of the enzyme-coding genes (Holton and Cornish, 1995; Mol *et al*. 1998; Wessinger and Rausher, 2012). For example, most variation in flower colour found in *Ipomea* can be attributed to insertion or deletion of transposable elements (Clegg and Durbin, 2000). Through the process of genetic drift, LOF mutations accumulate, which results in the degeneration of the branch or biosynthetic pathway and leads to pseudogenization or gene loss (Rausher, 2008; Ho and Smith, 2016). Once this degeneration has occurred it is almost impossible to restore the branch or pathway, as this would require multiple simultaneous mutations (Rausher, 2006).

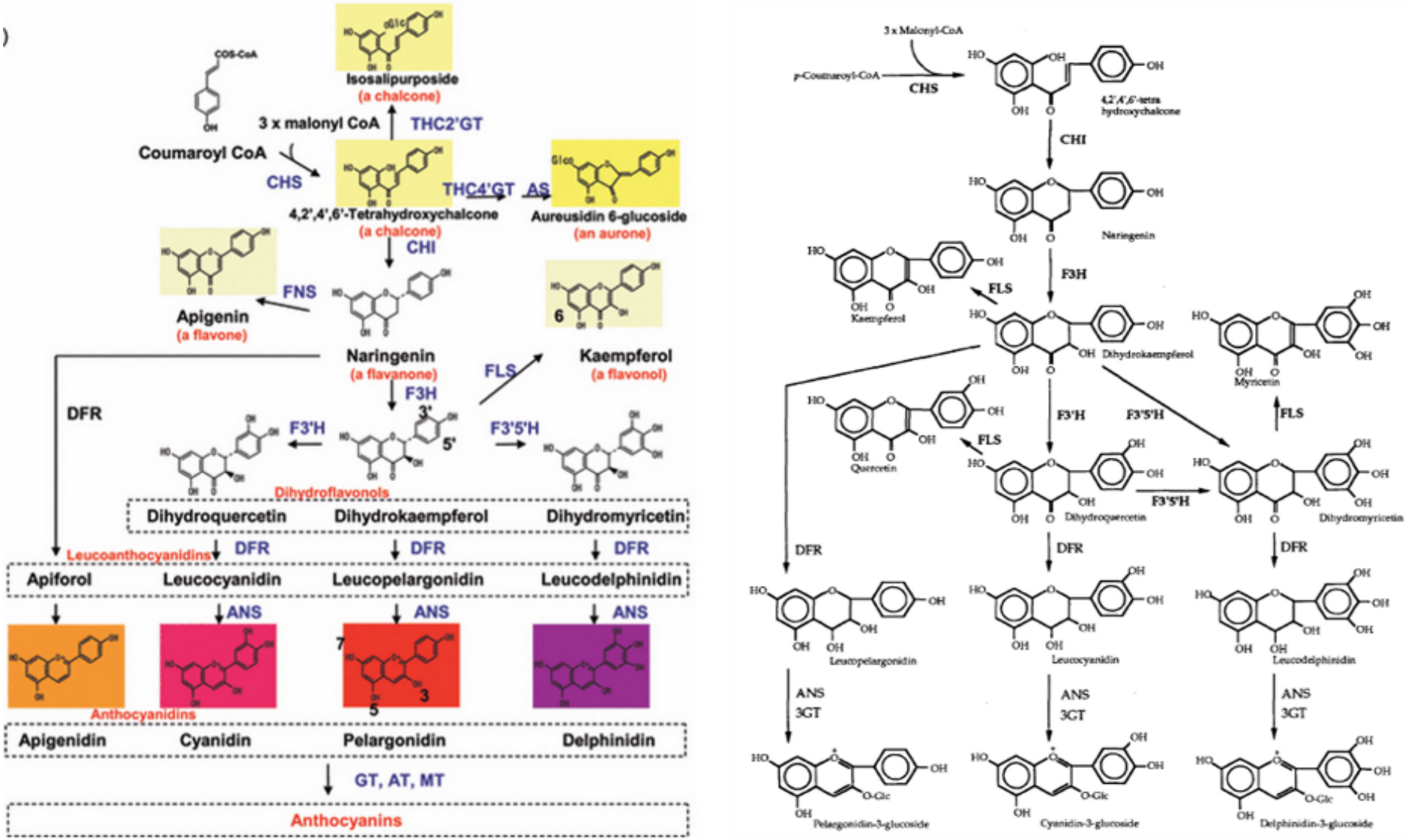

Figure 3.1. The anthocyanin biosynthetic pathway. **A.** Adapted from Tanaka *et al.* 2008 and **B.** Holton and Cornish, 1995. *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavonone 3-hydroxylase; *F3'H,* flavonoid 3'-hydroxylase; *F3'5'H*, flavonoid 3',5'-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase.

Rhodohypoxis baurii var. *confecta* (Hypoxidaceae) has naturally occurring polymorphisms (white, pink, and magenta morphs; Figure 3.2), which are derived from anthocyanin pigments (unpublished data). These morphs can be loosely grouped into pigmented (pink/magenta) and unpigmented (white) flower colour morphs (Figure 3.2). In this chapter the difference in ABP gene expression between pigmented and unpigmented *R. baurii* var. *confecta* morphs was investigated. This study focused on four of the ABP genes, *CHI*, *F3H*, *F3'H*, and *F3'5'H*, to determine if these genes are expressed differently between the two flower colour morph groupings (pigmented and unpigmented). In addition, the sequence similarity of the seven genes that make up the ABP was assessed in order to investigate whether this pathway is well conserved amongst angiosperm species.

It was expected that there would be a difference in gene expression between the unpigmented and pigmented *R. baurii* var. *confecta* morphs. As the pigmented morphs contain anthocyanin pigments that are most likely cyanidin derived (magenta/pink), it was expected that *CHI*, *F3H*, and *F3'H* would be expressed and *F3'5'H* would most likely not be expressed (no delphinidin pigments). On the other hand, unpigmented morphs do not appear to contain anthocyanin pigments and will most likely only have *CHI* expressed whereas *F3H*, *F3'H*, and *F3'5'H* will not be expressed.

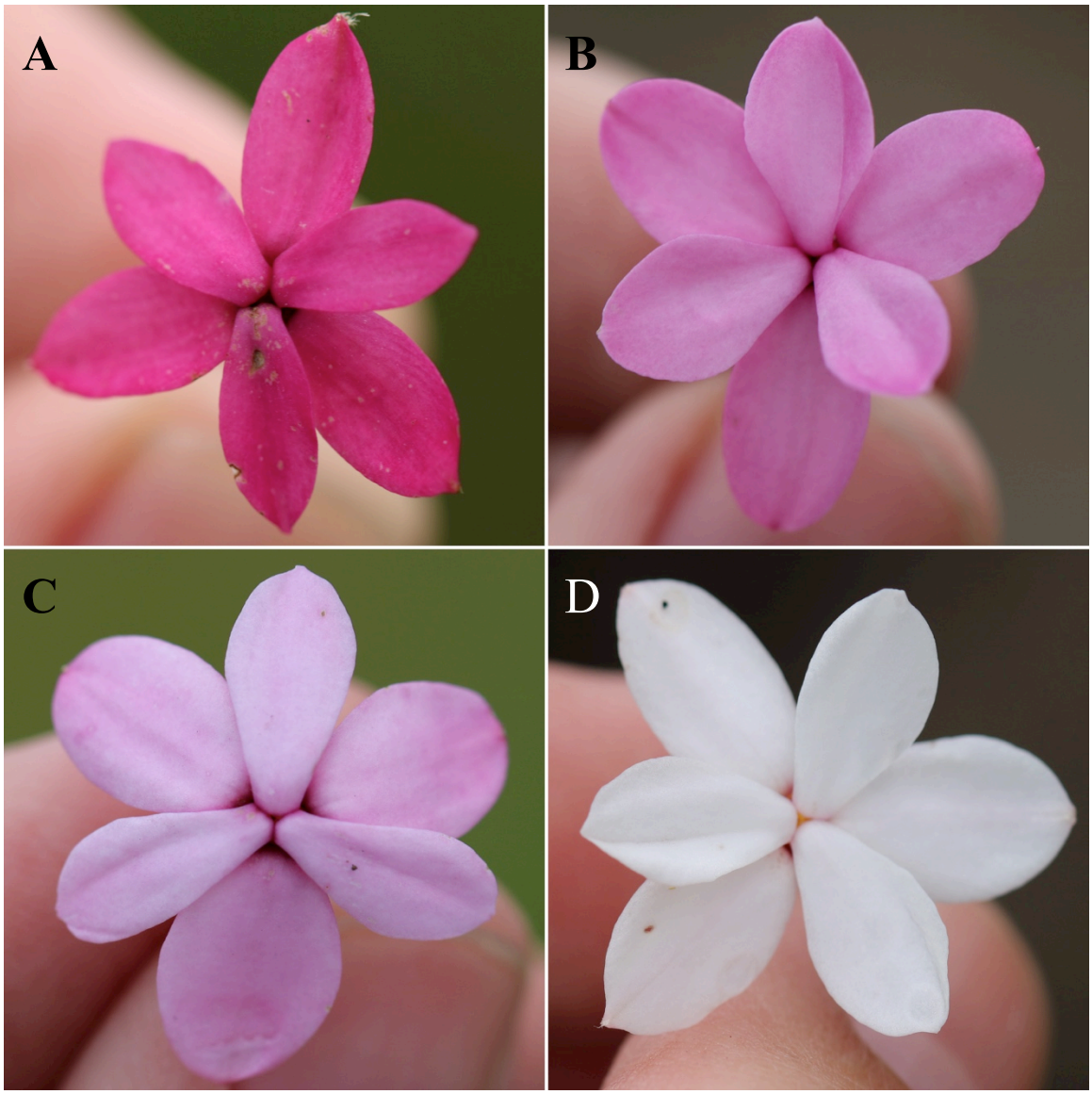

Figure 3.2. *Rhodohypoxis baurii* var. *confecta* flower colour morphs. **A** is a magenta flower, **B** is a pink flower, **C** is a light pink flower, and **D** is a white flower. **A, B** and **C** are pigmented morphs whereas **D** is an unpigmented morph.

Methods

Amplification success of cDNA for the four ABP genes, *F3H*, *F3'H*, *F3'5'H*, and *CHI*, (flavanone 3β-hydroxylase, flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase and chalcone isomerase) was compared between pigmented (pink/magenta), and unpigmented (white) flowers to examine patterns of expression in both morphs.

Primer design

Primers were designed to amplify the target regions for *R. baurii* var. *confecta* for four gene regions (*CHI*, *F3H*, *F3'H*, and *F3'5'H*; Table 3.1). Given that no Hypoxidaceae species have sequence resources available, sequences from other monocotyledons were used instead. Sequences for each target gene region were downloaded from GenBank (Table B.1 – Appendix B). Cultivar species, hybrid species, and partial sequences were excluded from the primer design. Sequences for each region were aligned using ClustalW implemented in Sequencher version 5.4.6 (Gene Codes Corporation, MI, USA). Consensus sequences were generated from each gene region's alignment and were used to design gene specific primers using Primer3 version 4.1.0 (Koressaar and Remm, 2007; Untergasser *et al.* 2012) under default parameters. Primers were then manufactured by Inqaba Biotec (Pretoria, Gauteng, South Africa).

Table 3.1. Primer sequences designed for four ABP genes (*CHI*, *F3H*, *F3'H*, and *F3'5'H*). Primers were designed using Primer3 (under default parameters) on consensus sequences generated for each gene region through the alignment of all available, and complete monocotyledon sequences (ClustalW in Sequencher version 5.4.6). Primer melting temperature (T_m) and GC content (%) are reported.

Gene region	Forward/ Reverse	Sequence (5'-3')	T_m (°C)	GC content (%)
CHI	Forward	CGATAGCGTGAAAACCCTGG	62.45	55
	Reverse	ATCGCTTTCCAATACGCCAC	60.4	50
F3H	Forward	GGCGCGAAATTGTGACCTAT	60.4	50
	Reverse	CTGCGGGCATTTCGGATAAA	60.4	50
F3'H	Forward	GAAACCTTTCGCCTGCATCC	62.45	55
	Reverse	GGCGAAATTCCAGCGGATC	62.32	57.89
F3'5'H	Forward	TACCAGCGCGATTGTGATTG	60.4	50
	Reverse	CAGCGCTTCTTTGCAAATCG	60.4	50

Sample collection

The genes amplified from floral cDNA were prepared from individuals that were collected from the Sentinel Car Park (SCP) population during each field trip between the October and December 2017 flowering season. A total of 20 unpigmented and 20 pigmented flowers (light pink, pink, medium pink, and magenta) were collected during this field season. White, light pink, pink, medium pink and magenta flowers were labelled as W, LP, P, MP and M, respectively. Floral tissue was removed from the stems with a sterilized scalpel and placed in 1.5ml microfuge tubes containing 650µl of RNA*later* Stabilization Solution (Thermo Fisher Scientific, MA, USA). Immediately after collection, samples were stored at 4°C overnight to allow the solution to thoroughly permeate the tissue. Samples were then stored at -20°C until RNA was extracted.

Total RNA extraction

Total RNA was extracted from approximately 0.04g to 0.09g of floral tissue from each single flower sample using the InviTrap Spin Plant RNA Mini kit (STRATEC Molecular, Berlin, Germany) following manufacturer's instruction, with several changes (see below). The outer petals were removed from the RNA*later* solution using sterile forceps approximately 12 hours prior to the RNA extraction process. When the petals were removed, it was weighed and any excess plant tissue that was not part of the petal was removed using a scalpel and discarded. The RNA*later* solution was discarded and excess solution that was still on the floral tissue was removed by blotting the flowers with tissue paper. The floral tissue was stored in the same microfuge tubes and frozen overnight at -20°C.

Several changes were made to optimize extraction yields. Frozen floral tissue was ground using stainless steel beads in a TissueLyser LT (Qiagen, Hilden, Germany). One bead was added to each sample tube and samples were disrupted for five minutes at 45rpm. Once the floral tissue had been ground into a fine powder, the beads were removed from each sample and 450µl of the Lysis Solution DCT was added to each tube. Samples were then shaken continuously in the TissueLyser LT for five minutes at 35rpm. Lysis solution foam was allowed to settle for one minute. These two steps were repeated an additional two times with the subsequent additions including 250µl and 200µl of the Lysis Solution DCT, respectively, and shaking continuously for five minutes at 35 rpm after adding each volume of lysis solution. Once a total of 900µl of the Lysis Solution DCT was added to each tube, the samples were vortexed for a total of five minutes at

one-minute intervals. The rest of the protocol was followed per the manufacturer's instructions. Samples were eluted with 40µl of Elution Buffer R.

The quality and quantity of total RNA in a 2µl volume was estimated for each sample using a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, MA, USA). A blank measurement was taken using 2µl of Elution Buffer R. Samples that had an RNA yield of more than 50ng/µl were synthesised into cDNA. First strand cDNA synthesis was done with 5µl of total RNA and 11µl of DNase-free water in a 20µl reaction using ReadyScript cDNA Synthesis (Sigma-Aldrich, Darmstadt, Germany). Following cDNA synthesis, the quantity of cDNA was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, MA, USA).

Amplification of target gene regions

A total of four flowers (two unpigmented and two pigmented, one medium pink, one magenta) were used to amplify target gene regions based on the quality and quantity of their cDNA. Initially, *CHI* was amplified using the ReadyScriptII cDNA as the PCR template. Each 10µl PCR reaction contained 50-100ng of the cDNA template, 5µl (2 U/µl) of *Taq* 2X Master Mix (New England BioLabs, MA, USA), 2.5µl of PCR-grade water, 1µM of the *CHI* forward primer, and 1µM of the *CHI* reverse primer. The PCR was run on a MyCycler Thermal Cycler PCR (Bio-Rad laboratories, CA, USA) as follows: 95°C for 2 min; 30 cycles of 95°C for 30 seconds, 55°C for 40 seconds and 72°C for 30 seconds; and finished with 72°C for 5 minutes and a 20°C hold. PCR products were run on a 1% agarose gel at 65 volts for 45 minutes in order to verify that a product had been amplified. A 100bp DNA ladder (New England BioLabs, MA, USA) with a size

range of 100bp to 1517bp was used in the gel. The *CHI* gene region did not amplify despite attempts to optimize annealing temperatures, $MgCl_2$ concentration, and cDNA concentration.

Amplification of target regions using the 3'RACE System

The 3'RACE System (Thermo Fisher Scientific, CA, USA) is used for the rapid amplification of cDNA ends (RACE) and is useful for instances where little sequence information is available. This system uses the natural Poly(A) tail found in mRNA as a generic priming site and captures 3'-mRNA sequences that lie between the targeted exon and Poly(A) tail by using gene specific primers and an adapter primer that targets the Poly(A) region.

The 3'RACE System protocol was used to test amplification of target gene regions in four individuals (two unpigmented and two pigmented) where RNA had been extracted from *R. baurii* var. *confecta* along with a control template that had been included in the kit. First strand cDNA synthesis was done with 11µl of total RNA due to the low starting concentrations of RNA and no additional DEPC-treated water was added to these samples.

A total of seventeen 3'RACE PCR reactions were conducted, consisting of two pigmented morphs and two unpigmented morphs for each of the four genes (*CHI*, *F3H*, *F3'H*, and *F3'5'H*) and a control. Each reaction had a final volume of 10µl and consisted of: 0.83µl 10X PCR buffer, 1.25mM $MgCl_2$, 6.08µl autoclaved-distilled water, 0.17mM dNTP mix, 0.17µM gene specific primer (GSP), 0.17µM abridged universal amplification primer (AUAP), 0.016 and 0.04 units/µl *Taq* DNA polymerase, and 50 to 100ng of the

cDNA template. Products were amplified on a MyCycler Thermal Cycler PCR (Bio-Rad laboratories, CA, USA) as follows: 95°C for 3 min; 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds; and finished with 72°C for 5 minutes and a 20°C hold. The PCR products were run on a 1% agarose gel at 65 volts for 45 minutes to verify that a product of an expected size had been amplified.

The *CHI* and *F3'H* 3'RACE PCR products were used as templated in a subsequent round of amplification as they had produced bands in the initial 3' RACE amplification. The other two regions (*F3H* and *F3'5'H*) were not successfully amplified, so were not considered for further investigation. A total of eight PCR reactions were conducted consisting of two pigmented morphs and two unpigmented morphs for each of the *CHI* and *F3'H* genes. Each reaction had a final volume of 25µl and contained: 10.5µl autoclaved-distilled water, 12.5µl Phusion *Taq* 2X Master Mix (New England BioLabs, MA, USA), 0.2µM forward primer, 0.2µM reverse primer and 1µl 3'RACE PCR amplicon as a template. The samples were run on a MyCycler Thermal Cycler PCR (Bio-Rad laboratories, CA, USA) as follows: 95°C for 30 seconds; 30 cycles of 95°C for 30 seconds, 62°C for 60 seconds and 68°C for 1 minute; and finished with 68°C for 5 minutes and a 10°C hold. The PCR products were run on a 1% agarose gel at 45 volts for 75 minutes. Post-PCR clean-up and sequencing of the *CHI* and *F3'H* PCR products was completed by the Central Analytical Facility at Stellenbosch University (Stellenbosch, Western Cape, South Africa). Chromatograms were edited by eye and analysed using MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 (Kumar *et al*. 2016). Regions of sequence similarity (somewhat similar sequences) were searched for on Basic

Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) using the sequence results to determine if the correct gene regions were amplified.

ABP genes alignment

Seven ABP gene sequences (*CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, and *ANS*) were compared across monocotyledon and dicotyledon species in order to understand how well conserved the ABP gene sequences are within angiosperms. Complete sequences of seven ABP genes (*CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, and *ANS*) were obtained from GenBank (Table B.2 - Appendix B). For this analysis, the complete gene sequence was used and the introns were excluded. These sequences were divided into two groups, monocotyledons and dicotyledons, for each of the seven ABP gene regions. The gene, *UF3GT,* was not included in this analysis as there were no complete monocotyledon and dicotyledon sequences available on GenBank. The monocotyledon, dicotyledon, and angiosperm (both monocotyledon and dicotyledon sequences) sequences were each aligned independently for each ABP gene region separately using MUSCLE implemented in Geneious 2018 (Kearse *et al.* 2012; https://www.geneious.com). These subsequent alignments were compared according to the percentage of identical sites, percentage pairwise identity, and by eye to infer how well conserved the ABP gene sequences are within angiosperms.

Results

Amplification of target regions using the 3'RACE System

CHI and *F3'H* were successfully amplified for both pigmented and unpigmented morphs (two pigmented and two unpigmented samples; Figure 3.3) however, one *F3'H* sample (W10) and two *CHI* samples (W19 and M2) produced non-specific smears (Figure 3.3). *CHI* amplicons produced a smeary band between 500 and 600 base pairs whereas *F3'H* ranged from 600 to 700 base pairs. *F3H* and *F3'5'H* were not successfully amplified for both pigmented and unpigmented morphs. The PCR products of *CHI* and *F3'H* were re-amplified using gene-specific forward and reverse primers (Table 3.1, Figure 3.4) and sequenced. Samples W10, MP2 and M2 for the *CHI* region produced non-specific smears but were still sequenced along with the other samples that had been re-amplified (Table B.3 - Appendix B).

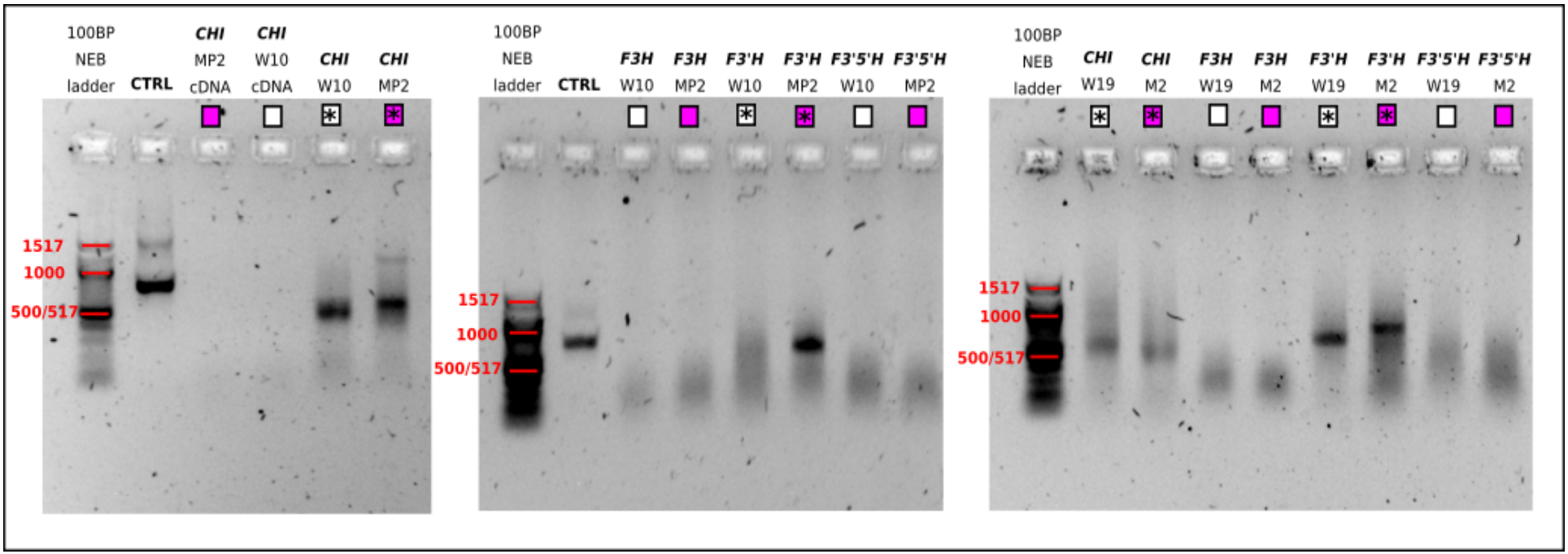

Figure 3.3. Amplification of target regions using the 3'RACE System. Four genes of the anthocyanin biosynthetic pathway (ABP) were amplified: chalcone isomerase (*CHI*), flavanone 3β-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*), and flavonoid 3', 5'-hydroxylase (*F3'5'H*). The samples were separated by electrophoresis on a 1% agarose gel. The sizes of the molecular weight standards are shown in the first lane of each gel (100 bp DNA ladder; New England BioLabs, MA, USA). A positive control (CTRL; 720bp) was used in the first and second gel (Lane 2) and no negative control was used. Pink and white squares represent pigmented (MP and M) and unpigmented (W) samples, respectively. Asterisks indicate the PCR products that were re-amplified and sequenced.

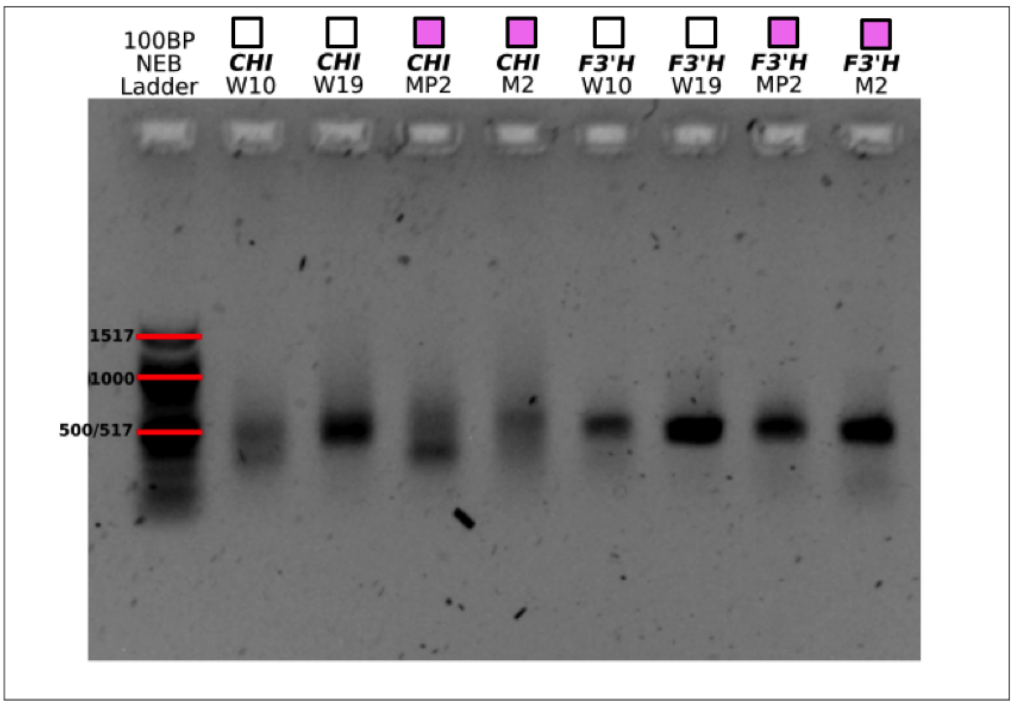

Figure 3.4. Re-amplification results of the chalcone isomerase (*CHI*) and flavonoid 3'-hydroxylase (*F3'H*) 3'RACE PCR products. Pink and white squares represent pigmented and unpigmented samples, respectively. The samples were separated by electrophoresis on a 1% agarose gel. The sizes of the molecular weight standards are shown in the first lane of the gel (100 bp DNA ladder; New England BioLabs, MA, USA).

The sequenced products were either clean sequence data (*F3'H*) or unclear sequence data (*CHI*) (Figure 3.5 and 3.6), but clean sequences did not match anticipated gene regions. Chromatograms and BLAST searches indicated that *CHI* amplicons appeared to have multiple templates amplified (Figure 3.5). *F3'H* was more specifically amplified for three out of the four samples (Figure 3.6). These sequences were BLASTed and search results indicated that the targeted gene regions, *CHI* and *F3'H*, had not been amplified (Table 3.2). Instead, a mixture of weakly matched search results spanning multiple phyla were returned for each sequence.

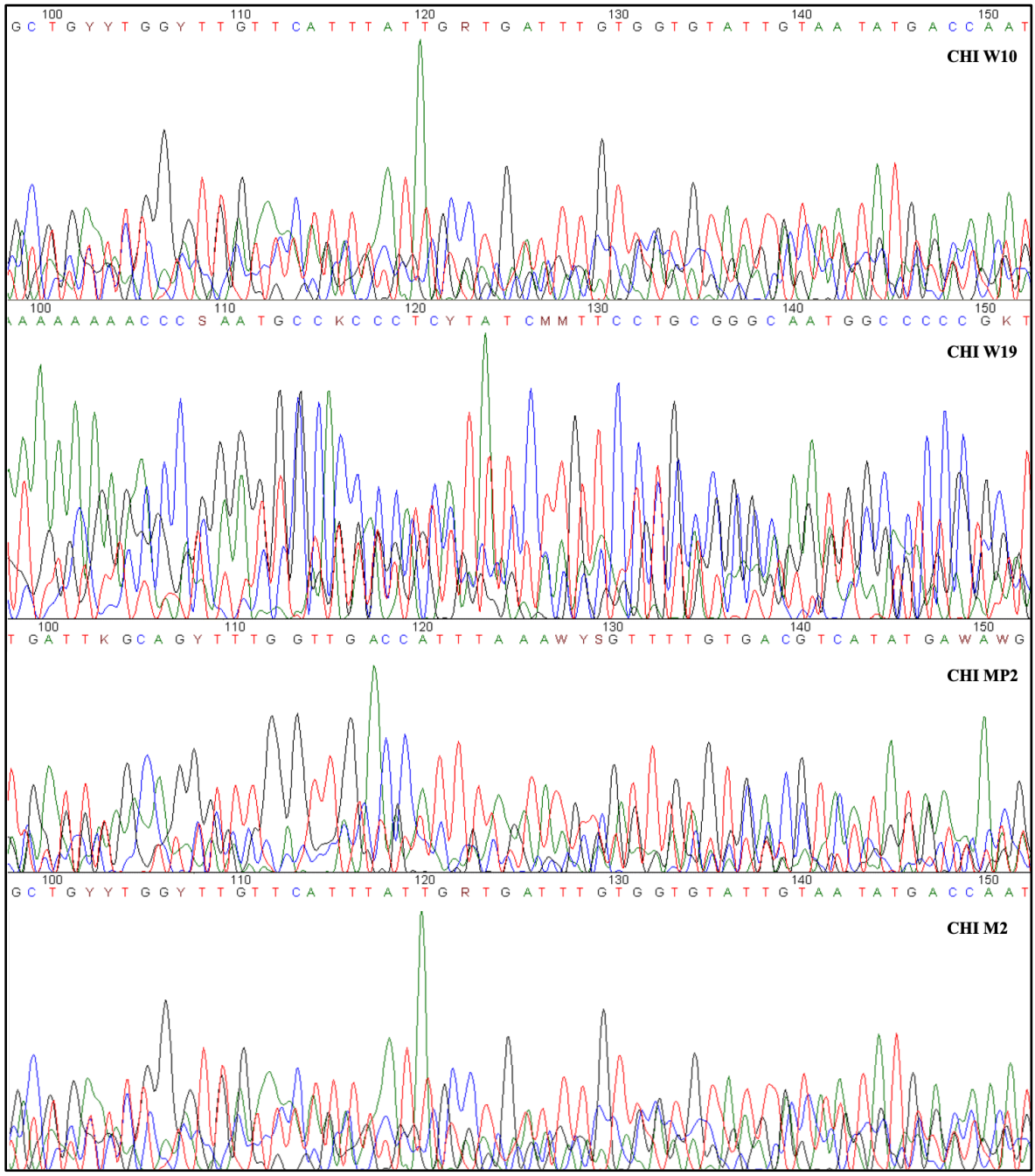

Figure 3.5. Chromatograms of the *CHI* region from between the 100 to 150 base pair mark for two unpigmented (white; W10, W19) individuals and two pigmented (pink; MP2, M2) individuals.

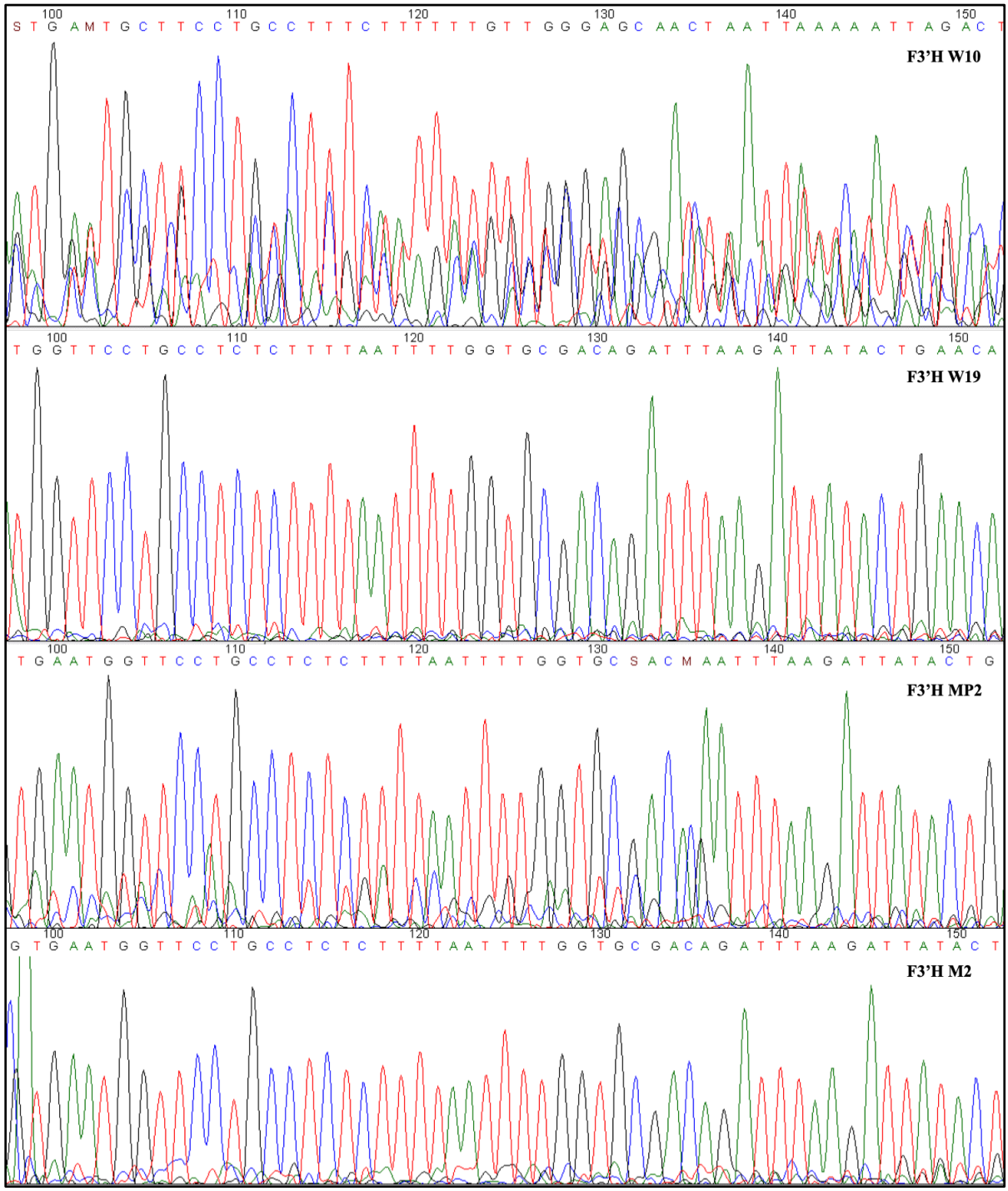

Figure 3.6. Chromatogram of the *F3'H* region from between the 100 to 150 base pair mark for two unpigmented (white; W10, W19) individuals and two pigmented (pink; MP2, M2) individuals.

Table 3.2. The top three BLAST search results of the sequenced *CHI* and *F3'H* PCR products. The search was optimized for 'somewhat similar sequences' (blastn) with default algorithm parameters.

Sample	Gene region	BLAST results
W10	*CHI*	***Nelumbo nucifera*** mitochondrion (KR610474.1) ***Liriodendron tulipifera*** mitochondrion (KC821969.1) ***Dioscorea rotundata*** mitochondrial DNA (LC219376.1)
W19	*CHI*	***Oryza sativa*** Indica Group cultivar Shuhui498 chromosome 2 (CP018158.1) ***Sinocyclocheilus rhinocerous*** protein AF-9-like (XM016543067.1) ***Pan troglodytes*** chromosome X clone CH251-244H4 (AC181984.1)
MP2	*CHI*	***Oryzias litipes*** strain Hd-rR chromosome 23 sequence (CP020687.1) ***Aedes albopictus*** secretory carrier-associated membrane protein 2 (XM019689383.1) ***Aedes albopictus*** secretory carrier-associated membrane protein 2 (XM019689382.1)
M2	*CHI*	***Lupinus angustifolious*** cultivar Tanjil chromosome LG-05 (CP023117.1) ***Homo sapiens*** chromosome 8 clone CTA-397H3 map q12.1 (AF165146.6) ***Homo sapiens*** chromosome 8 clone RP1-263I11 map q12.1 (AF191070.4)
W10	*F3'H*	***Scophthalmus maximus*** chromosome 8 (CP026250.1) ***Plasmodium coatneyi*** strain Hackeri chromosome 1 (CP016239.1) ***Plasmodium coatneyi*** strain Hackeri chromosome 6 (CP016244.1)
W19	*F3'H*	***Kluyveromyces marxianus*** strain NRRL Y-6860 chromosome 7 (CP023462.1) ***Vigna angularis*** **var.** ***angularis*** chromosome 2, cultivar: Shumari (AP025035.1) ***Vigna angularis*** **var.** ***angularis*** chromosome 11, cultivar: Shumari (AP015044.1)
MP2	*F3'H*	***Kluyveromyces marxianus*** strain NRRL Y-6860 chromosome 7 (CP023462.1) ***Danio rerio*** misshapen-like kinase 1 (mink1) (XM017356010.2) ***Danio rerio*** misshapen-like kinase 1(mink1) (XM017356008.2)
M2	*F3'H*	***Kluyveromyces marxianus*** strain NRRL Y-6860 chromosome 7 (CP023462.1) ***Danio rerio*** misshapen-like kinase 1 (mink1) (XM017356010.2) ***Danio rerio*** misshapen-like kinase 1(mink1) (XM017356008.2)

ABP genes alignment

The majority of sequences found for all seven ABP genes were dicotyledon species and there were fewer than ten monocotyledon sequences available for each of the ABP genes of interest. Overall many sequences were available for *CHS* (n = 38) and *DFR* (n = 20) however, more than 80% of these sequences were dicotyledon species. For all angiosperms (both monocotyledons and dicotyledons together), fewer than ten sequences were available for four of the seven ABP genes (*CHI*, *F3H*, *F3'H*, and *F3'5'H*). Only one monocotyledon sequence was available for *F3H* and *F3'H* and there were no monocot sequences available for *F3'5'H*.

Across all available angiosperms, *F3'5'H* had the greatest percentage of identical sites (65.8%) followed by *F3H* (50.7%). Other regions (*CHS*, *CHI*, *F3'H*, *DFR,* and *ANS*) had less than 35% identical sites within the compared angiosperms (Table 3.3). Pairwise identity within the gene regions *F3'5'H*, *CHS*, and *F3H* for all angiosperms was high (76.1%, 76.0%, and 73.1% respectively; Table 3.3). In contrast, pairwise identity within the other four regions within angiosperms was less than 64%. Sequence comparisons within dicotyledons showed a similar trend to the angiosperm comparisons. The percentage of identical sites and pairwise identity increased slightly for each gene region within dicotyledons (Table 3.4). *CHI* had the lowest pairwise identity (48.9% and 45.0%) and percentage of identical sites (9.7% and 15.3%) for all angiosperm sequences and dicotyledon sequences, respectively. The percentage of identical sites and pairwise identity was much higher in the monocotyledon sequences and *DFR*, *ANS*, *CHS,* and *CHI* had more than 50% identical sites and pairwise identity was consistently over 67% (Table 3.5). Within monocotyledon sequences, *DFR* had 84.4% pairwise identity and 69.9%

identical sites however, there were only four monocotyledon sequences available for this region.

Table 3.3. Comparison of sequence similarity of seven anthocyanin biosynthetic pathway (ABP) gene regions across 92 angiosperm sequences (both monocotyledon and dicotyledon sequences) that were available from GenBank.

Gene region	Number of Sequences	Pairwise Identity (%)	Identical sites (%)	Sequence length
CHS	38	76.0	33.8	1231
CHI	8	48.9	9.7	867
F3H	7	73.1	50.7	1153
F3'H	5	52.8	20.6	1587
F3'5'H	3	76.1	65.8	1535
DFR	20	63.7	25.5	1392
ANS	11	63.4	28.1	1389

Table 3.4. Comparison of sequence similarity of seven anthocyanin biosynthetic pathway (ABP) gene regions across 74 dicotyledon sequences that were available from GenBank.

Gene region	Number of Sequences	Pairwise Identity (%)	Identical sites (%)	Sequence length
CHS	31	78.6	43.3	1222
CHI	5	45.0	15.3	842
F3H	6	75.4	57.5	1137
F3'H	4	57.4	33.2	1569
F3'5'H	3	76.1	65.8	1535
DFR	16	66.4	29.5	1413
ANS	9	65.6	34.1	1374

Table 3.5. Comparison of sequence similarity of seven anthocyanin biosynthetic pathway gene regions across 18 monocotyledon sequences that were available from GenBank. Dashes represent instances where no further analysis was preformed because there was only one or no sequence available.

Gene region	Number of Sequences	Pairwise Identity (%)	Identical sites (%)	Sequence length
CHS	7	79.3	52.2	1209
CHI	3	70.7	59.4	721
F3H	1	-	-	-
F3'H	1	-	-	-
F3'5'H	-	-	-	-
DFR	4	84.4	69.9	1071
ANS	2	67.9	67.9	1095

Discussion

The aim of this study was to investigate whether there was a difference in ABP gene expression between unpigmented and pigmented *R. baurii* var. *confecta* morphs. To establish this the presence of gene expression in four genes in the ABP (*CHI*, *F3H*, *F3'H*, and *F3'5'H*) was tested in the two different morphs. It was expected that pigmented morphs would have *CHI*, *F3H*, and *F3'H* expressed, whereas *F3'5'H* would most likely not be expressed in these morphs. In unpigmented morphs it was expected that only *CHI* would be expressed and not the other three ABP genes. *CHI* and *F3'H* appeared to be amplified for both pigmented and unpigmented morphs, whereas *F3H* and *F3'5'H* were not amplified. In addition, the similarity of the genes within the ABP was assessed in order to determine if the sequences in the ABP pathway are well conserved.

The primers used for the *CHI* gene were non-specific, and amplified an off target product in both pigmented and unpigmented morph samples. The expression of *CHI* was

expected, but not found. It was expected that *CHI* would be expressed as it is upstream of pigment production and is directly responsible for the production of flavones and isoflavones (Tanaka *et al*. 2008), as well as indirectly responsible for the production of other products further downstream such as tannins, and anthocyanins which, play important plant physiological roles particularly in terms of environmental stress response and pollinator attraction (Winkel-Shirley, 2001; Sobel and Streisfield, 2013). If *CHI* is not expressed the production of these products will be prevented and the rest of the pathway will be shut off. This has been demonstrated in the flowers of *Ipomoea alba* L. (Rausher, 2006) and in some *Solanaceae* vegetables such as tomatoes and peppers (Borovsky *et al*. 2004; Sapir *et al.* 2008; Stommel *et al.* 2009; Povero, *et al.* 2011; Aza-Gonzalez *et al.* 2013) where *CHI* is expressed equally in pigmented and unpigmented morphs. However, there are some instances where the concentration of anthocyanin pigments correlate positively with the level of expression of *CHI*, for example in other *Solanaceae* vegetables such as, eggplants and potatoes where the level of expression of *CHI* differs between unpigmented and pigmented morphs (De Jong *et al.* 2003; Jung *et al.* 2005; André *et al.* 2009; Jung *et al*. 2009; Zheng *et al.* 2009; Stushnoff *et al*. 2010; Payyavula *et al.* 2013; Liu *et al.* 2015; Stommel and Dumm, 2015; Gisbert *et al.* 2016; Jiang *et al*. 2016).

The results from sequencing the *CHI* region indicated that the *CHI* gene region had not been amplified. The chromatograms for each *CHI* sample were unclear, which could suggest that more than one region had been amplified. In addition, BLAST search results did not result in *CHI,* but rather traced back to a broad range of different phyla and gene regions. This was most likely because the gene specific primers that had been

designed were not specific enough to amplify the desired gene region. Since the designed primers for *CHI* were not specific enough to amplify *CHI* in *R. baurii* var. *confecta,* it is possible that the *CHI* monocotyledon sequences used to design the primers are perhaps polymorphic and the sequences may not be as well conserved as current literature suggests.

Overall *CHI* does not appear to be well conserved among all the angiosperm species sequences and this trend is evident across the dicotyledon species in particular. In the angiosperm and the dicotyledon groups, *CHI* has the lowest pairwise identity score and percentage of identical sites when compared to the other six ABP genes. These scores are much higher in the *CHI* monocotyledon sequences, however this could be because there are only three *CHI* monocotyledon sequences available to compare. These low similarity scores explain why *CHI* was not successfully amplified with the designed primers.

The primers used for the *F3'H* gene, were non-specific, and amplified an off target product in both pigmented and unpigmented morph samples. *F3'H* was expected to be expressed for the pigmented morph samples, as *F3'H* is directly responsible for the production of cyanidin pigment, which produces pink/red flower colour and is most likely the dominant pigment in pink/magenta *R. baurii* var. *confecta* flower morphs. Unpigmented (white) flowers lack pigment and as a result an unpigmented *R*. *baurii* var. *confecta* morph should theoretically have no anthocyanin pigments. If pigment is not being produced then there is a possibility that *F3'H* would not be expressed however, *F3'H* may be expressed but non-functional or it is possible that genes further downstream in the ABP (e.g., DFR, and ANS) are not expressed and as such are responsible for the

lack of pigment production. Some unpigmented *R. baurii* var. *confecta* individuals experience localised pigmentation and sometimes have slight tinges of pink underneath their petals or display pink, during their development, in the budding stages and as a result *F3'H* may be expressed in these morphs. The expression of the ABP genes have been found in other studies to differ both spatially and temporally, with localised areas of pigmentation and colour variation occurring at different stages of plant growth respectively (Falginella *et al.* 2012; Xie *et al.* 2015). For example, the ABP genes in unripe strawberries with white tissue have much lower expression levels than ripe strawberries with red tissue (Salvatierra *et al.* 2010). It is also possible that unpigmented *R. baurii* var. *confecta* morphs have anthocyanin pigments in their vegetative tissue. Although unpigmented morphs appear to lack anthocyanin pigments in their floral tissue, some white *Ipomoea purpurea* L. morphs have been found to contain anthocyanin pigments in their vegetative tissue as a result of a mutation in a tissue-specific regulatory gene (Coberly and Rausher, 2003).

The amplified *F3'H* PCR products were also sequenced along with the *CHI* amplicons. The resulting chromatograms for each *F3'H* sample were cleaner than the chromatograms produced for the *CHI* samples and had more defined peaks with less noise. Although the chromatograms were ideal, the *F3'H* amplicon sequences did not match any existing *F3'H* sequences on the BLAST database. From these results, it is clear that much like *CHI*, the *F3'H* gene region had not been amplified and that this was also most likely a result of non-specific primer design.

All available angiosperm sequences for *F3'H* were compared and were found to have relatively low similarity scores (Table 3.3). *F3'H* had the second lowest pairwise

identity score and the sequences had a very low percentage of identical sites when compared to the six other ABP genes. Moreover, this same trend was observed in the dicotyledon sequence group. Overall there was a lack of full sequences available for *F3'H* and only one full monocotyledon sequence and four full dicotyledon sequences were found on the NCBI database. This lack of sequence data could be as a result of fewer studies focusing on the *F3'H* gene region relative to other ABP genes.

The other two targeted ABP genes, *F3H* and *F3'5'H*, did not produce any bands and appeared to have not been successfully amplified for both the unpigmented and pigmented morph samples. This result was unexpected for *F3H* as it is upstream of *F3'H* and needs to be expressed in order for *F3'H* to be expressed (Holton and Cornish, 1995; Tanaka *et al.* 2008). It is likely that these results are due to a false negative as a result of non-specific primer design rather than a lack of *F3H* and *F3'5'H* expression. *F3H*, *F3'H*, and *F3'5'H* dictate the structure and type of pigments (pelargonidin, cyanidin, and delphinidin) that are produced by flowers (Tanaka *et al*. 2008; Tanaka and Brugleira, 2013). Based on this knowledge, it was expected that *F3'5'H* would not be expressed in pigmented *R. baurii* var. *confecta* morphs, as they do not appear to contain delphinidin (blue/purple) pigments. However, it is also possible that either *F3'5'H* is expressed but non-functional or the genes downstream of *F3'5'H* (e.g., *ANS* and *DFR*) are not expressed and therefore delphinidin pigments are not produced. In addition, it was expected that there would be a marked difference in gene expression for *F3H* between pigmented and unpigmented morphs. Differences in gene expression between pigmented and unpigmented morphs have been found in some *Solanaceae* species where both *F3H* and *F3'5'H* were found to have a positive correlation with anthocyanin content

(Borovsky *et al.* 2004; André *et al*. 2009, Stushnoff *et al.* 2010, Povero *et al*. 2011; Aza-Gonzalez *et al.* 2013). Contrary to what was expected, results appear to show that *F3H* was not expressed in pigmented morphs. Based on these results it appears that *F3H* and *F3'5'H* had also not been amplified however, it is possible that this was a false negative.

The similarity of both *F3H* and *F3'5'H* were each assessed and were found to have relatively high similarity scores compared to the other ABP genes that had been analysed. For both the angiosperm and dicotyledon analyses, the pairwise identity scores for both *F3H* and *F3'5'H* were relatively high and approximately 20% higher than the scores for *CHI* and *F3'H*. This was also reflected in the percentage of identical sites scores, for which *F3'5'H* and *F3H* had the highest scores compared to all other ABP genes within dicotyledon and angiosperm species. These high similarity scores could be a consequence of the lack of sequence data available. This is particularly evident with the monocotyledon sequences where only one, if any, sequence was available.

As the molecular work conducted on the targeted ABP genes was unsuccessful and the genes of interest were not amplified, it is likely that the primers that were designed for this molecular work were not specific enough to amplify the target gene regions. As no sequence data were available for *Rhodohypoxis* or Hypoxidaceae, monocotyledon species were used to design the primers for the genes of interest under the key assumption that the ABP genes are well conserved among angiosperm species (Table B.1 - Appendix B; Holton and Cornish, 1995; Rausher, 2006; Tanaka and Ohmiya, 2008). When comparing all available angiosperm sequences for these four gene regions we found that the gene regions were not as similar as we had believed and in some cases, specifically for *CHI* and *F3'H*, sequences were highly variable and appeared to be

polymorphic. In order to fully assess if sequences from other species are good options for primer design, the similarity of the other three gene regions in the pathway (*CHS*, *DFR*, and *ANS)* was quantified.

CHS is the key enzyme of flavonoid biosynthesis and is situated at the beginning of the pathway (Liu *et al*. 2018). *DFR* and *ANS* are found at the end of the pathway and influence both anthocyanin composition and pigmentation (Liu *et al.* 2018). There were many sequences available for *CHS*, whereas there were fewer sequences available for *DFR* and *ANS*. Overall, there are at least 80% more dicotyledon sequences compared to monocotyledon sequences available for *CHS*, *DFR*, and *ANS*. There is a clear disparity between the number of full monocotyledon and dicotyledon sequences and the number of dicotyledon sequences available are always much larger than the number of monocotyledon sequences available. Pairwise identity scores and the percentage of identical sites for *ANS*, and *DFR* were found to be relatively low while *CHS* had very high similarity scores despite a large sample size. When analysed in each of the dicotyledon and monocotyledon groups, both pairwise identity scores and the percentage of identical sites scores increase substantially for all three gene regions however, this could be a consequence of the lower sample size. Most notably, the four *DFR* monocotyledon sequences have substantially higher similarity scores compared to all the *DFR* angiosperm sequences together.

It appears that some of the ABP genes are polymorphic and as a result the sequences of these genes are not well conserved. *CHI* and *F3'H* have very low similarity scores in all three groupings followed by *DFR* and *ANS,* which also have relatively, low similarity scores. *CHS*, *F3H*, and *F3'5'H,* on the other hand, have higher similarity

scores and the sequences appear to be more well conserved. The lack of sequence data available could have an effect on the accuracy of these similarity scores especially in the cases of *F3'5'H*, *F3'H*, *F3H*, and *CHI*. In addition, there are a disproportionate number of studies that focus on the ABP genes in dicotyledon species compared to those that include monocotyledon species and in some cases no more than one monocotyledon sequence could be found (*F3H*, *F3'H*, and *F3'5'H*). This is most likely because monocotyledons are a small group of plants that make up only 25% of the world's flowering plant species whereas the dicotyledons are a larger, and more diverse group with more evidence of intra-specific flower colour variation (Fay, 2013).

One possible explanation for the variability among sequences across species could be that the basic structure and function of the ABP is well conserved, but the gene sequences themselves are polymorphic. The ABP evolved in multiple steps over time (Rausher, 2006). *CHS*, *CHI*, and *F3H* were the first genes to develop in the pathway and formed the backbone of the ABP (*CHS-CHI-F3H*) (Rausher, 2006). The *CHS-CHI-F3H* backbone evolved early in the evolutionary history of land plants (Marchantiophyta and Bryophyta) and these genes were responsible for the production of flavanones, flavones, and flavonols. *F3'H* and *F3'5'H* were recruited next and helped form the three major branches of the pathway. These genes were recruited into the pathway through the duplication of genes in the cytochrome P450 hydroxylase family (Dixon and Steele, 1999). *DFR* and *ANS* were the last two additions to the flavonoid pathway and occurred much later in evolutionary history. *DFR* was recruited in the Filicophyta and was responsible for the production of proanthocyanidins (Rausher, 2006). *ANS* was recruited

in the flavonoid pathway when gymnosperms and angiosperms evolved and marked the beginning of the production of anthocyanins.

The evolution of the ABP could provide some insight into the conservation of the genes that make up this pathway. *CHS* and *F3H* may have high similarity scores because they were the first genes recruited into the ABP and have been a part of the pathway since the emergence of terrestrial plants (Rausher, 2006). *CHS* and *F3H* form two-thirds of the backbone of the pathway and so it is possible that the function of these genes have remained relatively well-conserved in the pathway. On the other hand, *DFR* and *ANS* were recruited into the pathway much more recently and *ANS* is only found in angiosperms. As a result, *DFR* and *ANS* have been a part of the ABP for a much shorter period of time, which could be why these genes are more variable and more dissimilar. Conversely, *CHI* also formed part of the initial backbone of the ABP and *F3'H* and *F3'5'H* were recruited in the ABP at the same time as *F3H* and *CHS*. These three gene regions (*F3'H*, *F3'5'H*, and *CHI*) however, do not show the same pattern as what was expected from their evolutionary history. *CHI* and *F3'H* had very low similarity scores whilst *F3'5'H* had a relatively high similarity score. It is also possible that the low similarity scores for *CHI*, *F3'H*, *DFR*, and *ANS* reflect a lack of sequence data and perhaps these scores would increase if more sequence data were available.

Alternatively, the many functions of the ABP may explain the patterns of similarities and dissimilarities found between gene regions in the pathway. The ABP is pleiotropic and is not only responsible for the production of anthocyanins, but also a number of other flavonoids including, flavones, flavanones, and flavonols. *CHS*, *CHI*, and *F3H* are directly responsible for the production of these compounds, which are

related to plant fitness and defence (Chalker-Scott, 1999; Grotewold, 2006; Ryan *et al*. 2002; Tanaka *et al.* 2008). These compounds are found in most terrestrial species and as a result the functions of these genes have most probably remained the same. The bottom half of the ABP pathway (*F3'H*, *F3'5'H*, *DFR*, and *ANS*) is more directly responsible for the production of anthocyanin pigments and more specifically determines the type of anthocyanin pigment produced (Chalker-Scott, 1999; Grotewold, 2006). Flower colour variation is largely dictated by varying patterns of expression, levels of expression and in some cases the inactivation of some ABP genes. For example, pelargonidin anthocyanins are not produced in *Petunia* hybrids Juss. and *Cymbidium* hybrids Swartz*,* as *DFR* in these species cannot utilize dihydrokaempferol (Tanaka *et al*. 2008). In some instances certain ABP gene regions are not found in some species, for example *F3'5'H* is not found in roses (*Rosa* hybrids L.), carnations (*Dianthus caryophyllus* L.), and chrysanthemums (*Chrysanthemum morifolium* Ramat.) and as a result they do not produce delphinidin anthocyanins (Tanaka *et al*. 2008). On the other hand, some species of Asteraceae have reacquired *F3'5'H* though convergent evolution (Seitz *et al*. 2006). In different angiosperms, the genes directly responsible for pigment production (*F3'H*, *F3'5'H*, *DFR*, and *ANS*) are often modified or are not present in certain plant species. In contrast the ABP genes that occur further upstream (*CHS*, *CHI*, *F3H*) are more directly responsible for the production of the other compounds that benefit plant health and as a result are almost always present and are not modified.

In a broader context, perhaps the ABP gene sequences are not as well conserved as expected because the genes are under a number of different selection pressures, which vary between species. The entire ABP is not only responsible for the production of

anthocyanin pigments, but many other compounds. These compounds aid in plant disease defence, pollen viability, microbial interactions, trichome density, root hair formation, cell morphology, vacuolar size, and UV protection (Koes *et al*. 1994; Mol et al., 1993; Walker *et al*. 1999; Spelt *et al*. 2002; Winkel-Shirley, 2002; Morita *et al*. 2006). Due to its pleiotropic nature the ABP could therefore be under different selective pressures associated with many of its multiple functions. In addition, flower colour itself is under multiple selection pressures. Anthocyanins are primarily associated with flower colour, however, they are also associated with environmental stress response (Koes *et al*. 1994; Mol *et al.* 1998; Walker *et al*. 1999; Spelt *et al*. 2002; Winkel-Shirley, 2002; Bradshaw and Schemske, 2003; Morita *et al*. 2006). As a result, anthocyanins can be under both pollinator and non-pollinator mediated selection (e.g., Levin and Brack, 1995; Galen, 2000; Coberly and Rausher, 2003; Gomez, 2003; Strauss *et al.* 2004). A similar phenomenon has been observed in a vertebrate immune complex, the major histocompatibility complex (MHC), where the complex is well conserved, but is highly polymorphic (Ejsmond and Radwan, 2015). The MHC is one of the most variable loci in the vertebrate genome (Kelley *et al*. 2005) as a result of the combined effects of selective forces that act on the pathway in different scenarios including pathogen-mediated selection (natural selection), and disassortative mating (sexual selection) (Ejsmond and Radwan, 2015). Despite the complex being highly polymorphic across vertebrate species, the overall structure of the complex has remained intact. It is possible that a similar scenario is occurring in the ABP, which appears to be, highly polymorphic across angiosperm species, but still retains the same function and overall structure.

Conclusion

The difference in ABP gene expression between unpigmented and pigmented morphs in *R. baurii* var. *confecta* (*CHI*, *F3H*, *F3'H*, and *F3'5'H*) was investigated. The primers used for the *CHI* and *F3'H* genes were non-specific, and amplified an off target product in both pigmented and unpigmented morph samples, whereas *F3H* and *F3'5'H* did not produce bands in all samples for both morphs. This was contrary to the expectation of a clear difference in gene expression of *F3H* and *F3'H* between unpigmented and pigmented morphs. These results were most likely due to non-specific primer design as primers were designed using sequences from other monocotyledon species. The similarity of the ABP genes were then analysed in order to assess if sequences from other species remain good options for primer design. This would also inform if the ABP gene sequences are as well conserved as the literature suggests. The results from this similarity analysis indicated that the gene sequences in the ABP are polymorphic and most likely not well conserved amongst angiosperm species, but rather that the overall structure and function of the pathway is well conserved. The sequences that make up the ABP were found to be polymorphic most likely as a result of differential selective pressures acting on the different traits in the pathway under different conditions which has been found to occur in pathways such as the major histocompatability complex (MHC; Ejsmond and Radwan, 2015). Further work is needed to reassess the difference in ABP gene expression between unpigmented and pigmented *R. baurii* var. *confecta* morphs using primers designed from *Rhodohypoxis* or other Hypoxidaceae species. In addition, it would be worthwhile further investigating the conservation of the ABP in order to understand how different selection pressures act on polymorphic gene sequences in the ABP.

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CHAPTER FOUR

GENERAL CONCLUSION

The study of flower colour has proved to be a particularly valuable approach to investigating fundamental evolutionary questions (Clegg and Durbin, 2000). Flower colour is a phenotype that is an easily measured expression of the plant genotype and it provides a strong indicator of the outcomes of selection pressures. The biochemistry and genetics of flower colour are well understood (Harbone, 1988; Stafford, 1990; Forkmann, 1991; Quattrocchio *et al.* 1993; Holton and Cornish, 1995; Mol *et al*. 1993; Chalker-Scott, 1999; Winkel-Shirley, 2002; Grotewold, 2006; Tanaka *et al.* 2008). Consequently, molecular and ecological methods can be used in combination to understand flower colour both by understanding the genes responsible for determining this phenotype as well as the environmental selection pressures acting on this phenotype. The overall aim of this study was to investigate the maintenance of flower colour using *Rhodohypoxis baurii* var. *confecta* as a study system. This involved understanding the genetic mechanisms that regulate flower colour (Chapter Three) and examining the environmental variables that drive this trait variation across populations (Chapter Two).

Hilliard and Burtt (1978) postulated that individual *R. baurii* var. *confecta* flowers change colour over the flowering season and open as white flowers which become gradually pink and then magenta over their lifespan. Through monitoring individual plants, this study confirmed that populations of *R. baurii* var. *confecta* display true polymorphisms of magenta, pink, and white and that individual flowers remain the same colour throughout the flowering season. In addition, individual plants consistently

produce the same coloured flowers, either pigmented (pink/magenta) or unpigmented (white) flowers, throughout the flowering season.

Within populations of *R. baurii* var. *confecta* the proportion of these polymorphisms (pigmented and unpigmented morphs) have been found to change within a single flowering season. At one population in this study, the proportion of pigmented (pink/magenta) morphs flowering increased significantly over the flowering season and the population shifted from a predominantly unpigmented flowering population at the beginning of the flowering season to a predominantly pigmented population flowering by the end of the flowering season. Other populations did not show the same shift in the proportion of unpigmented and pigmented morphs and maintained a relatively high proportion of unpigmented morphs throughout the flowering season. Long-term studies have found shifts in the proportion of flower colour morphs over multiple flowering seasons (e.g. Schemske and Bierzychudek, 2001, 2007; Warren and Mackenzie, 2001; Arista *et al*. 2013; Veiga *et al*. 2016) however, no studies were found that documented short-term changes in the proportion of polymorphisms within a population over a single flowering season.

Soil moisture was identified as the environmental variable that could potentially drive the flower colour variation and colour frequency shift observed within this *R. baurii* var. *confecta* population. The increase in the production of pigmented morphs correlated with increasing levels of soil moisture over the flowering season. In those populations where the proportion of unpigmented and pigmented morphs remained relatively the same, soil moisture never reached the same high levels as the population where the colour frequency shift took place. It is possible that environmental variables, specifically

soil moisture, trigger the production of pigmented morphs. Many studies are now exploring environmental drivers of flower colour variation (e.g. Levin and Brack, 1995; Warren and Mackenzie, 2001; Coberly and Rausher, 2003; Carlson and Holsinger, 2015) some of which have pinpointed water availability as a selection agent for flower colour (Galen 2000; Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001). However, these studies found that pigmented morphs were favoured in environments with low moisture availability (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Strauss and Whittall, 2006). This study does not coincide with these findings as pigmented morphs occur more frequently when soil moisture is at its highest. Studies conducted at a finer scale and long term monitoring of *R. baurii* var. *confeta* would be required to confirm and expand on findings presented here.

At a molecular level, this study attempted to examine whether differences in flower colour are caused by differential expression in the ABP by determining if there is a difference in gene expression between unpigmented and pigmented *R. baurii* var. *confecta* morphs. As there was no sequence data available for the study system or closely related taxa (Hypoxidaceae), the primers were designed by aligning multiple monocotyledon sequences. This primer design method was used as literature has cited that the ABP is well conserved amongst all angiosperm species (Quattrocchio *et al.* 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al*. 2014). The molecular work for this component of the study was however unsuccessful as the designed primers were found to be not specific enough to amplify the gene regions of interest. This inexact primer design raised broader questions about the conservation of the ABP.

As a result further investigation of the ABP gene sequences was carried out in order to assess if it was well conserved amongst angiosperm species. Overall the gene sequences that make up the ABP appeared to not be as well conserved as the literature had suggested (Quattrocchio *et al.* 1993; Holton and Cornish, 1995; Winkel-Shirley, 2002; Rausher, 2006; Tanaka *et al*. 2008; Campanella *et al*. 2014). This was particularly evident in some gene regions (e.g., *CHI* and *F3'H*) more than others (e.g., *CHS* and *F3H*). The basic structure of the ABP appears to be well conserved however the actual gene sequences specifically may not be as well conserved. This could be as a result of the numerous selective pressures acting on flower colour such as both the non-pollinator and pollinator selection agents. In some angiosperm species flower colour is predominantly under selection from pollinators (e.g. Giurfa *et al*. 1995; Lunau and Maier, 1995; Morgan and Schoen, 1997; Niovi Jones and Reithel, 2001; Wessinger and Rausher, 2012; Alcalde-Eon *et al.* 2013), however flower colour in other angiosperm species is under selection from environmental factors (e.g. Levin and Brack, 1995; Warren and Mackenzie, 2001; Coberly and Rausher, 2003; Carlson and Holsinger, 2015). These selective pressures act on the pathway in different ways and have a direct effect on plant survival (non-pollinator mediated selection) and reproductive success (pollinator mediated selection). As the type and extent of these selection pressures vary across angiosperm species and act on these species in different ways it could explain the polymorphic nature of individual gene region sequences within the ABP across angiosperm species.

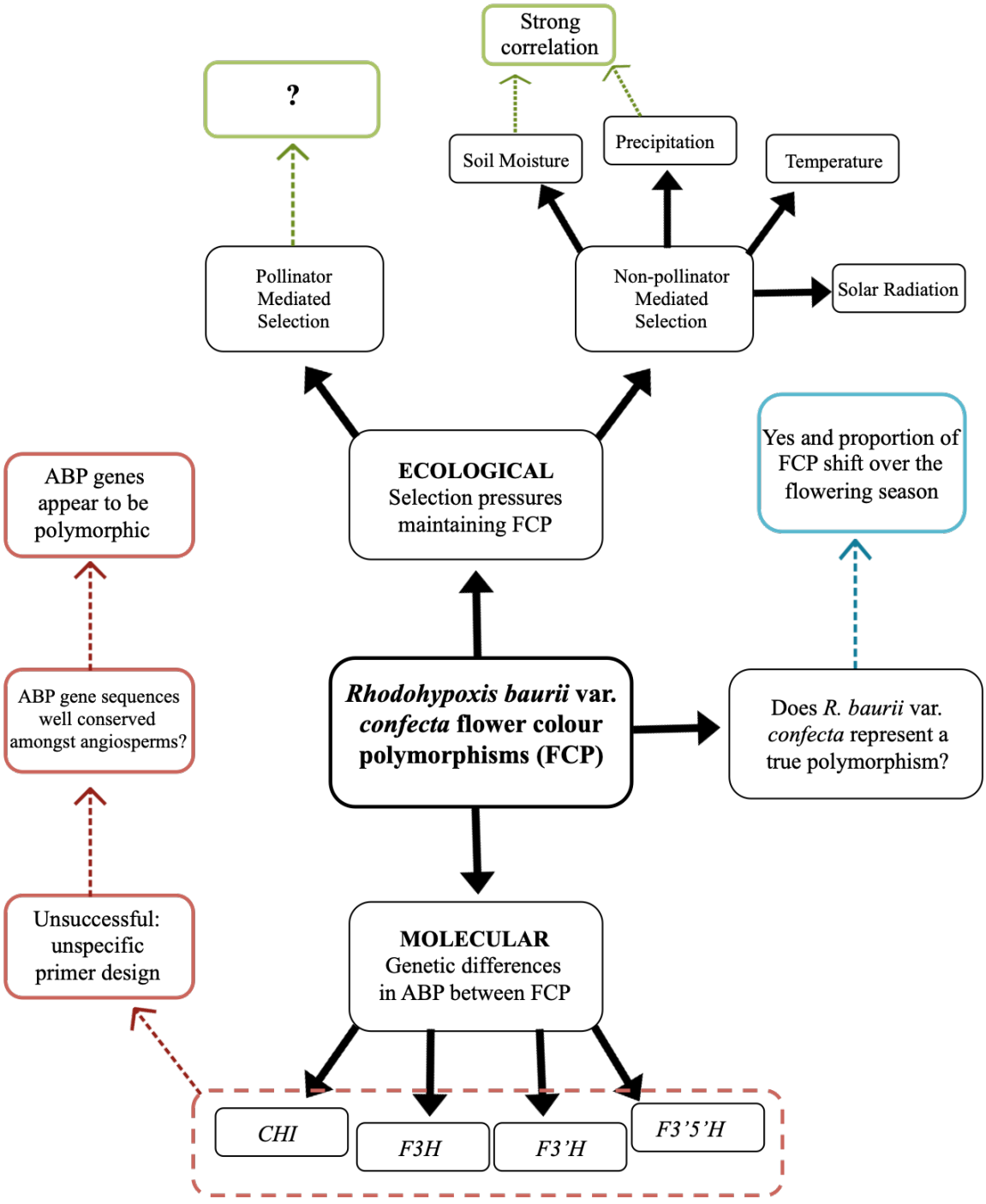

Figure 4.1. A synthesis of the results of this study. Findings of this study are in coloured boxes.

Recommendations for future work

This study has provided foundational work on the maintenance of *Rhodohypoxis baurii* var. *confecta* colour polymorphisms. However, research can be expanded on and pursued in the future.

ABP gene expression in unpigmented and pigmented flower colour morphs

This study was unsuccessful in assessing whether genes in the ABP were expressed differently in unpigmented and pigmented *R. baurii* var. *confecta* morphs. However, findings from this study concerning the conservation of the ABP will inform future molecular work to be done on the ABP and on *R. baurii* var. *confecta* specifically. In order to readdress this objective, specific gene sequence data from either *Rhodohypoxis* or the broader Hypoxidaceae is required to design specific primers to amplify the genes of interest. This specific gene sequence data could be obtained through next-generation DNA sequencing. This extends to molecular work done on the ABP in other angiosperm species, particularly in monocotyledon species where there is generally limited sequence data available. There is a disproportionate number of dicotyledon ABP sequences available, relative to the number of monocotyledon ABP sequences, and more work is needed to address this disparity. *R. baurii* var. *confecta* (a monocotyledon species) would be a good species to start with when addressing this gap.

Rhodohypoxis baurii varieties

The *Rhodohypoxis baurii* complex is not well understood and other than Hilliard and Burtt's initial description (1978) and work currently being conducted aims to better

eludicate the evolutionary history and evolutionary trajectory of this genus and specifically the *R. baurii* varieties (Glennon, unpublished data). Based on Hilliard and Burtt's (1978) description, only *R. baurii* var. *confecta* occurs around the Sentinel Peak area. In this study, two of the populations maintained a high proportion of unpigmented morphs with few very light coloured pigmented flowers. This is similar to *R. baurii* var. *platypetala*, which is described as having populations that contain predominantly white flowers with few instances of light pink morphs. As the varieties in the *R. baurii* complex are largely differentiated by flower colour and habitat, it would be worth investigating if these two populations are made up of *R. baurii* var. *confecta* or *R. baurii* var. *platypetala*. Alternatively, *R. baurii* var. *platypetala*, *R. baurii* var. *confecta,* and *R. baurii* var. *baurii* are described as occurring in dry, damp, and moist habitats, respectively and are thus largely differentiated by the amount of soil moisture in their habitat (Hilliard and Burtt, 1978). It is also worth investigating whether *R. baurii* is a highly variable species where the production of pigmented flowers is favoured in areas with high soil moisture (as described in this study). It is possible that the environment in which *R. baurii* occurs results in local adaptation and influences the flower colour of the population (pink/magenta or white) and whether the population is polymorphic (*R. baurii* var. *confecta*) or mostly monomorphic (*R. baurii* var. *platypetala* and *R. baurii* var. *baurii*).

Reproductive biology of Rhodohypoxis baurii var. confecta

There is still much to learn about the reproductive strategy of *R. baurii* var. *confecta*. Biyela *et al.* (unpublished data) has found that individuals are capable of both sexual and asexual (vegetative) reproduction and are self-compatible. No pollinators have been found to come into contact with the reproductive parts of the flower during the day

and as a result pollinators were not considered as flower colour selection agents (pers obs). *R. baurii* var. *confetcta* flowers are small (10 –16mm wide) and its reproductive organs are well hidden by its petals so pollinators would have to be small in order to burrow into the flower to come into contact with pollen. More extensive pollinator observations at varying times of the day (especially at night) would be necessary to further confirm that no pollinators interact with the flowers.

Long-term monitoring at Sentinel Peak population

Long-term flower colour frequency and soil moisture measurements would be extremely valuable in confirming the trends established in this study. Schemske and Bierzychudek (2001) collected fitness, pollinator, and precipitation data at three populations of *Linanthus parryae* over the course of 11 years. Through doing this they were able to draw conclusions about temporal variation in selection and how it contributed to the maintenance of flower colour polymorphisms within populations of *L. parryae* (Schemske and Bierzychudek, 2001). If the same populations of *R. baurii* var. *confecta* are monitored over the next few years using the pre-existing transects then we can establish if the populations undergo the same shift in flower colour that was observed in this study. In addition, we can further confirm whether this shift in flower colour corresponds and is maintained by changes in soil moisture over the flowering season. Similarly to Schemske and Bierzychudek (2001), fitness data (seed counts) and counts of the proportion of flowering plants should also be collected during this fieldwork in order to determine if there is a difference in plant fitness between unpigmented and pigmented morphs. This data will aid in further understanding why pigmented flowers are favoured

in periods of high soil moisture when the opposite is true in other studies (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Strauss and Whittall, 2006).

Growth chamber experiments

The role of soil moisture in maintaining flower colour polymorphisms in *R. baurii* var. *confecta* can be further investigated by conducting growth chamber experiments. Under controlled conditions, the amount of water plants receive can be manipulated in order to determine if soil moisture truly influences the colour of the flower that is produced. From this data we could determine if there is a soil moisture threshold value that cues particular morphs to flower. In addition, in the greenhouse the same plant can be monitored over multiple flowering seasons in order to determine if the same plant continues to produce the same flower colour in different flowering seasons.

Spatial variability at Sentinel Peak population

Future work can be done to look at the variability of soil moisture at a finer scale. By measuring the spatial variation of soil moisture and the occurrence of pigmented flower colour morphs on a small-scale (e.g., every 1m along the transect) we could further test whether pigmented flower production corresponds to high soil moisture levels. In this study, soil moisture measurements were highly variable within a single transect whereas other environmental variables such as solar radiation, temperature, and precipitation were not variable over a small-scale but varied temporally. Through measuring the spatial variability of soil moisture we can test whether spatial variation in soil moisture has the same effect on flower colour frequency as temporal variation in soil

moisture (over the flowering season). This would further support that soil moisture is the driving variable of flower colour variation in *R. baurii* var. *confecta*.

ABP gene expression in leaves of pigmented and unpigmented morphs

It is also worthwhile to look at the difference in ABP gene expression in the leaves of unpigmented and pigmented *R. baurii* var. *confecta* morphs. Mtileni et al. (unpublished data) found that there was no difference in anthocyanin content when using a Flame spectrometer (Ocean Optics, Inc.) to measure the reflectance of the leaves of the two different *R. baurii* var. *confecta* morphs. It may therefore be beneficial to include both the leaves as well as the floral tissue in further molecular analysis when comparing unpigmented and pigmented flower colour morphs. Some white *Ipomoea purpurea* morphs lack anthocyanin pigments in their floral tissue but have anthocyanin pigments in their vegetative tissue as a result of a mutation in a tissue-specific regulatory gene (Coberly and Rausher, 2003). A similar phenomenon may be occurring in the leaves of unpigmented *R. baurii* var. *confecta* morphs.

Studying flower colour using different plant species

Several species have been used as model species for the study of flower colour polymorphisms and have been studied in considerable depth. Some of these model species include, *Ipomea* L. (e.g. Ennos and Clegg, 1983; Epperson and Clegg, 1986, 1988; Austin and Huáman, 1996; Glover *et al.* 1996; Coberly and Rausher, 2003), *Petunia* (e.g. Hagendoorn *et al*. 1991; Quattrocchio *et al*. 1999; Johnson *et al*. 2008), and *Phlox* (e.g. Levin and Kerster, 1967; Levin and Brack, 1995; Hopkins and Rausher, 2011; 2014). The study of these model species has shed light on the maintenance of

polymorphisms and the genetic underpinnings of such polymorphisms. However, it is important to study a variety of different plant systems, including monocotyledonous species, in order to build on our knowledge of flower colour polymorphisms. *R. baurii* var. *confecta* displays one of the first known instances where a population shifts flower colour within a single flowering season. In addition, this species has been found to produce pigmented flowers in periods of high water availability, which is not consistent with current literature (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Strauss and Whittall, 2006). This is also one of few studies involving both a monocotyledon species (e.g. Mudalige-Jayawickrama *et al.* 2005; Feller *et al*. 2011; Lai *et al*. 2012) and looks at environmental drivers of flower colour variation in a South African context (e.g. Carlson and Holsinger, 2015). It is important to compare and contrast research done on model study systems (e.g. *Ipomea*, *Petunia*, and *Phlox*) with novel studies on lesser-known species to see if the same findings hold true in different contexts.

Conclusion

Understanding what maintains flower colour both on an ecological and molecular level is important as this is a highly variable phenotypic trait that has the potential to increase biodiversity. The Drakensberg near-endemic *R. baurii* var. *confecta* populations have naturally occurring polymorphisms of white, pink, and magenta flowers. The frequencies of these polymorphisms were found to change within a single flowering season and these changes in colour corresponded closely with changes in soil moisture. As a result, it is possible that these polymorphisms are maintained by non-pollinator as opposed to pollinator mediated selection. Investigation of the genetic mechanisms that

regulate the flower colour polymorphisms in *R. baurii* var. *confecta* was most probably unsuccessful as a result of non-specific primer design. If the primer design was non-specific it raises questions about the conservation of the ABP gene sequences across angiosperm species.

The study demonstrates results that are not consistent with other research on different species. It further contributes to understanding how flower colour polymorphisms are maintained in natural populations and is the foundational work in understanding the polymorphisms of *R. baurii* var. *confecta*. Future work will further investigate and readdress the molecular component of this work, and will assess other non-pollinator mediated selection agents such as solar radiation as a potential driver of flower colour polymorphisms.

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APPENDIX A

Appendix for Chapter Two

Table A.1. The AIC and ΔAIC values for the 15 possible model combinations used for the generalized linear mixed models for all three populations. Models were ranked according to their ΔAIC values and the model with the lowest ΔAIC was determined to best fit the colour frequency data. 'Soil moisture' represents the recorded daily minimum soil moisture, 'Temperature' represents recorded minimum daily temperature and 'Solar radiation' represents monthly average solar radiation values.

	K	AICc	Δ AIC
Soil Moisture and Julian date	4	964.89	0.00
Julian Date, Temperature, and Soil Moisture	5	965.45	0.56
All (Global)	6	965.81	0.91
Temperature, Soil Moisture, and Solar Radiation	5	966.70	1.80
Julian Date, Temperature, and Solar Radiation	5	978.37	13.48
Solar Radiation and Temperature	4	980.38	15.48
Julian Date and Temperature	4	981.37	16.47
Solar Radiation and Soil Moisture	4	981.70	16.81
Temperature and Soil Moisture	4	981.92	17.02
Julian Date	3	984.86	19.97
Solar Radiation anf Julian Date	4	985.26	20.36
Soil Moisture	3	1015.09	50.54
Temperature	3	1029.24	53.54
Solar Radiation	3	1029.24	64.35

Null	2	1277.10	312.21

APPENDIX B

Appendix for Chapter Three

Table B.1. Monocotlyedon sequences and their accession numbers that were used to design primers for the four ABP genes of interest (*CHI*, *F3H*, *F3'H*, and *F3'5'H*). Consensus sequences were generated by aligning sequences using ClustalW on Sequencher and primers were designed using Primer3.

Gene region	Species	Accession number
CHI	*Zea mays*	Z22760
	Triticum timopheevii	KJ000522
	Tricyrtis sp.	AB908277
	Tulipa fosteriana	KC261502
	Tulipa fosteriana	KF792731
	Lycoris radiata	KF732852
F3H	*Zea mays*	U04434
	Tricyrtis sp.	LC209222
	Dioscorea alata	KF561995
	Anthurium andraeanum	DQ972935
	Curcuma alismatifolia	GU140081
F3'H	*Tricyrtis hirta*	AB480691
F3'5'H	*Dendrobium moniliforme*	HQ412560

Table B.2. Monocotyledon and dicotyledon sequences used for the alignment of seven genes in the anthocyanin biosynthetic pathway (ABP). Dashes indicate when no sequence data was available.

Gene region	Monocotyledon/Dicotyledon	Species	Accession Number
CHS	Dicotyledon	*Prunus persica*	JN391444
		Fagopyrum dibotrys	GU169470
		Fagopyrum tataricum	GU172165
		Litch chinensis	GU288820
		Rorippa amphibia	AF144530
		Arabidopsis himalaica	AF144531
		Cochlearia danica	AF144532
		Arabidopsis korshinskyi	AF144533
		Lepidium campestre	AF144534
		Thlapsi arvense	AF144535
		Alliaria petiolata	AF144537
		Cardamine penzesii	AF144538
		Cardamine rivularis	AF144539
		Cardamine pratensis	AF144540
		Sisynbrium irio	AF144541
		Ionopsidium abulense	AF144542
		Solanum tuberosum	HQ659493
		Olea eurpaea	KF935223
		Arabis jacquinii	AF112097
		Arabis lignifera	AF112098
		Arabis lyallii	AF112099
		Arabidopsis lyrata	AF112100
		Capsella rubella	AF112106
		Barbarea vulgaris	AF112108

		Aubrieta deltoidea	AF112109
		Hibiscus cannabinus	KJ605650
		Antirrhinum maius	X03710
		Vitis vinifera	AB015872
		Ipomoea purpurea	AB004905
		Senna tora	JX676773
		Halimolobos perplexa var. perplexa	AF112094
CHI	Dicotyledon	*Olea europaea*	KF886190
		Morus alba	JQ824050
		Erythranthe lewisii	KJ011134
		Actinidia chrysantha	KM453235
		Solanum tuberosum	HQ659497
F3H	Dicotyledon	*Glycine max*	EU391459
		Glycine soja	EU391458
		Nicotiana sylvestris	MF445074
		Nicotiana tomentosiformis	MF445073
		Arabidopsis thaliana	U33932
		Brassica napus	DQ288238
F3'H	Dicotyledon	*Glycine max*	AB191404
		Ipomoea purpurea	AY333419
		Ipomoea purpurea	AY333420
		Dianthus caryophyllus	AB778537
F3'5'H	Dicotyledon	*Vitis vinifera*	AB213606
		Glycine max	EF174665
		Glycine max	EF174666
DFR	Dicotyledon	*Arabidopsis thaliana*	AB033294
		Fragaria pentaphylla	KR075887
		Erythanthe lewisii	KJ011136
		Liquidamber formosana	JX975398

		Punica granatum	KP726344
		Brassica rapa	AY953249
		Fagopyrum cymosum	EF522146
		Solenostemon scutellaroides	EF522156
		Lotus corniculatus	AY633707
		Solanum tuberosum	HQ659495
		Saussurea medusa	EF672626
		Iochroma calycinum	JN593314
		Iochroma baumii	JN593317
		Iochroma parvifolium	JN593318
		Dunalia brachyacantha	JN593319
		Dunalia solanacea	JN593320
ANS	Dicotyledon	*Solanum cardiophyllum*	HQ701727
		Brassica juncea	EU927146
		Erythranthe llewisii	KJ011137
		Liquidambar formosana	JX975399
		Solanum melongena	KT727966
		Punica granatum	KP726345
		Nicotiana tomentosiformis	MF445067
		Nicotiana sylvestris	MF445068
		Arabidopsis thaliana	JF681791
CHS	Monocotyledon	*Oryza sativa*	AB058397
		Thinopyrum ponticum	AY286094
		Hordeum vulgare	X58339
		Secale cereale	X92548
		Curcuma longa	AB573022
		Tulipa fosteriana	KJ935033
		Triticum aestivum	AY286096
CHI	Monocotyledon	*Hordeum vulgare subsp. vulgare*	AF474923

		Canna generalis	DQ160231
		Zea mays	Z22760
F3H	Monocotyledon	*Anthurium andraeanum*	DQ972935
F3'H	Monocotyledon	*Dracaena cambodiana*	MG675627
F3'5'H	-	-	-
DFR	Monocotyledon	*Triticum aestivum*	AY210885
		Lophopyrum ponticum	AY298997
		Triticum aestivum	AY208998
		Anthurium andraeanum	DQ364060
ANS	Monocotyledon	*Anthuriu andraeanum*	EF079869
		Tulipa fosteriana	KJ935032

Table B.3. Sequence results for eight samples that were successfully amplified for the *CHI* and *F3'H* gene regions of the anthocyanin biosynthetic pathway (ABP). Samples starting with W were unpigmented/white samples and samples starting with M and MP were pigmented samples.

Sample	Gene	Sequence
M2	***CHI***	CGCCAAACATTAATTTCCCTTCTCTTATTATGCGTAGCTCCTTGCTCCC TGTGGCCGCTTACACTTCAAAACTGTTTCGTTGACTTCGTTTTGATTGG CTGATTGGATTGTACATTTATAGCTGATTTGTTGTGTATTGTAATATGA CAAATATTTCTTTATTTCGGTTGGTGTTTGTATGCCTGAAACGCAATTC CGCCCTGTATTTTATCTCGATGAGATCACCCCGAGTGTCAATCTGCAA TGAGTGATTTGAGATGTTCTGTGTGATATGATTAATGCTTTCTGATGCT ATTGTAAATCTGTATATCATTTGCCTATGTTCTTCATCAACTTTCTTTG ACGTAATTGCTTTCCTGTTTCCCTTTACCACTAATTTTATTCGAATGTT ATCCTGTTTAGGTTTTAGGGTGAGAAGTTTGGGGGTGTGTTGGTGGTA CGGTGGGTGAAGGTTGGTGGGTGGGTGAGCTCTCAATTTCAAGAACA AGAAGACTTGTTCCTTGAGTGTARTATTGGAAAGCGATTTCCTTSTTGG KTTG
MP2	***CHI***	TCAATTTGGGTTTTACGTAGCTACTCTGATCTAGATCGTGAACTCCTAG AGATTCCTCTACCCCCTTGTTTTCATACAAATTTCGATTACCTCATTTTT GATTKGCAGYTTTGGTTGACCATTTAAAATCGTTTTGTGACGTCATAT GAAACGACCAATATTTTTTTTTTTCGTTTAGTTTTGCATTTTTGGTTCCC CATTCTGGAGCGGTATTTTTTTTTTTTTTTTAGACTACCGGGGAAACTG AAAAAAGCAAATTTGTTGGGGGTTACGAAAATTGSAAGGTGAATATCT AACCCTAAGTGGGGTATTGACAAGCGACCACCCACGGGGTGCCTTCTC CCTCCAACCCTTGGGTCTTCTRACATATACAGTGAATTGGACTGAAAG GGCTTCCC
W10	***CHI***	CCGTAGCTTAATGATCGCGACTCGCTCTATTTCTACGAGCTCCAGGGG ATGCCTCTACCGAGTAGTTTTCATGCATAGTTCGATTAGCTCATTTTTA ATTTAAAGTTTTGGTTGCCCATTTAGTTGGGTTTTGTGATGTATTATAC CCCCGACCAATATTTTTTTATTTCGTTTGGTTTTGCATTTTTGGTTCACC ATTCTGTTGCGTATTTTTTTTCTTTTTTTTCCGCGAAATGATGAACAGA AAAAATCCATTTTGTTCATTAATTACGCCTATTGGAAGGYGAATGGGA AGCTGTAAGTGGCGTATTGGAAAGCGATGAYAAATTGGCTAACTAGT TTTGGCAAGCACCCAAATCTACGATTTTAAATATGTTCATGTACTGGTT TTGTTATCTGGGGGCGTTTTGGAAAGCAAATATCCACTCATTCTTGAT ACTATCAGTCGAAGTCMCGGCGTGGGGGGACTCGAAATAACAACGTC GTGGCGTATTGGAAAACAATAATACTATAT
W19	***CHI***	GACGGTGCAGGATGAAAGGTTCTGAGTGGAGGATATGTTGGCCAGAA CTACTTCACCTAGTGCTTATATCAACCTAATGCGAGTTCGGATCTCAA AAAAAAAAAGCCCGAGTGCCCCCCTCATATCTTTTCCTGCGGGCAATG GCCCCCGTTTCCGAAAGGTTGAAAATTTTTCCCCGGGTTTTGCCCGAA GGCCAGGTTCTCGGGGGAACCCACCACCCCTAAAACCCCCCCCCCGCA TGGGTCTAATGAAAAAATTTGTTTGAAAAATTAAAACCCTTCTCAAGC CCTCTGGACCTTGTTTGTTTTTTTTCCCATTTGCCTATKTTATTGGTCAA CTTTGTTTGGGGTAATTGKAAATCCTGAATTTCTAAAAAATTMATTTT ATTCGAATGTTATCCGGGGGSGGTTTGAAAAAGAAAAAATTGGGGGT GTGTTGGTGGTACGGTGGGTGAAGGTTGGTGGGTGGSTGAGCTCTCAA TTTGAGGAAKCTTGAAAACTTATTCCTTGAGTGTCGTATTGGAAAGCG A
M2	***F3'H***	GCATGACCAGCTGCTCTATAGYTTCCACTTCTGATGACAAAGTATGAA ACWTCCCCATCCACTTAAATTTTTACCTGGAATCTGGATTTTCCCCCTG GTGAATGGTTCCTGCCTCTCTTTTAATTTTGGTGCGACAGATTTAAGAT TATACTGAACAGTTCAGGAGGTAGTGGAGCTAACATTTCTGTTCATAT TCTGATTTGGGGTGCAATGGATTAAGAATATTGGAGGGTGGAGTAAA GACTTGGGATCAATTTTTTGGTGGGATGCAGCAAAAATGGAGACAAA AGGCTCCTAAAGGGAGTTGATGTTGCTTCTGCCTCTTCAGTGTCTTCTG CCTTTTGCCATTGGAGTTGGGCAGCAGAGGAAGGCCTTAGAACTGACA TAAATGGTCTCCAATGCGTGACCTGTTGATCCGCTGGAATTTCGCCA

MP2	***F3'H***	CGCGTGCAAGCTGTTTTACTGCTTCACTTCCTGATGTCAAGGAGGAAA CATCCCCATCCACATAGATAGATAGCTGGAATCTGGAGTTTCCCCCTG GTGAATGGTTCCTGCCTCTCTTTTAATTTTGGTGCGACMAATTTAAGAT TATACTGAACAGTTCAGGAGGTAGTGGAGCTAACATTTCTGTTCATAT TCTGATTTGGGGTGCAATGGATTAAGAATATTGGAGGGTGGAGTAAA GACTTGGGATCAATTTTTTGGTGGGATGCAGCAAAAATGGAGACAAA AGGCTCCTAAAGGGAGTTGATGTTGCTTCTGCCTCTTCAGTGTCTTCTG CCTTTTGCCATTGGAGTTGGGCAGCAGAGGAAGGCCTTAGAACTGACA TAAATGGTCTCCAATGCGTGACCTGTTGATCCGCTGGAATTTCGCC
W10	***F3'H***	GGGCATTACAAACTGTTTATTGCATCAACGTCTGATGTCAAGGASGAA ACATCCCCATCCACATAGATAGCTCTCTTCAGTCTGTACTTCCCCCCTG ATGAMTGCTTCCTGCCTTTCTTTTTTTTTGGGAGCAACTAATTATTAAT TAGACTGAACAGTTAAGGAGGGGATGTAGCTAACCTTTCCGTTCATAT ACTGAGGGGGGGCGCAATGAATAAAAAAGAAKGGAGGATGAAAGAA ATGCTTGCRATTTTTTTTTGGGAGGSATGCAACATGGATACAAACRGA TCGTTCCTGGAGGTAATGTATGTTCCGCCTCCCCCTTGTCTGCCGTCTT TCGTTTGCCAATGTGGTTGGCCAACAAAGGACTGCCATATAACTTACA TGGWCGCCCTCCAATGACCGGCCTGTCCATCCGATTTACTTCCGCCA
W19	***F3'H***	AARRRAACCACTGTTTTATTGCMTCAACGTCTGATGTCAAGGAGGAAA CATCCCCATCCACATAGATAGCTGGAATCTGGAGTTTCCCCCCGGTGA ATGGTTCCTGCCTCTCTTTTAATTTTGGTGCGACAGATTTAAGATTATA CTGAACAGTTCAGGAGGTAGCGGAGCTAACATTTCTGTTCATATTCTG ATTTGGGGTGCAATGGATTAAGAATATTGGAGGGTGGAGTAAAGACT TGGGATCAATTTTTTGGTGGGATGCAGCAAAAATGGAGACAAAAGGC TCCTAAAGGGAGTTGATGTTGCTTCTGCCTCTTCAGTGTCTTCTGCCTT TTGCCATTGGAGTTGGGCAGCAGAGGAAGGCCTTAGAACTGACATAA ATGGTCTCCAATGCGTGACCTGTTGATCCGCTGAAATTTCGCAAA