THE BIOLOGY OF POLLINATION AND SEED DISPERSAL IN *CLIVIA* (AMARYLLIDACEAE)

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Centre for
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FOR
MY PARENTS
AND GRANDPARENTS

"*We must consider the distinctive characters and the general nature of plants from the point of view of their morphology, their behaviour under external conditions, their mode of generation and the whole course of their life"*

Theophrastus, Enquiry into Plants (*Historia Plantarum*)

ABSTRACT

Pollinator shifts have been implicated as drivers of angiosperm diversification. The hypothesis that a transition from bird to butterfly pollination took place in *Clivia* was tested, and floral traits investigated to determine which may have mediated the putative shift. Linking pollination systems with available phylogenies indicated a shift occurred from bird to butterfly pollination, accompanied by the evolution of upright trumpet-shaped flowers, scent emission and nectar volume reduction, whilst floral colouration and nectar chemistry remain unmodified. Results support the idea that pollinator shifts may explain major floral trait modifications during plant diversification. Breeding systems of *Clivia* were investigated, with the aims of demining the site and functional consequences of putative late-acting self-incompatibility (LSI). Results suggest that *Clivia* species are largely self-sterile as a result of LSI or severe inbreeding depression, but ovule discounting caused by self-pollination is not a major limitation on fecundity, and seed production appears to be mostly resource limited. *Clivia miniata* is pollinated virtually exclusively by butterflies. Functional significance of *C. miniata* floral traits were examined, with the aim of determining butterfly floral preferences and the functional basis of traits responsible for butterfly pollination. Colour is a key advertising signal, with orientation facilitating alighting, whilst size, scent, and shape also influence butterfly attraction. Dispersal mechanisms of numerous fleshy seeded Amaryllidaceae have been an enigma as seeds are unorthodox, toxic and unable to survive ingestion, yet packaged in brightly coloured fruits suggestive of animal dispersal. Dispersal and germination of *Clivia miniata* seeds was investigated. Results indicated consumption of fruit by primates which disperse seeds through non-ingestive spitting behaviour. The short distance of seed dispersal by primates is predicted to lead to restricted gene flow and genetic subdivision of populations. I conclude that shifts in pollination systems and the associated modification of suites of functional floral traits led to floral diversification in *Clivia*. Self-infertility in *Clivia* highlights pollinator dependence and pollination syndrome conformity reflects functional advertising signals. Gene flow appears to be governed by pollen flow and facilitated by pollinators rather than seed dispersal. Mating and breeding system evolution are likely a consequence of adaptation to isolated forest habitats.

PREFACE

The experimental work described in this dissertation was conducted by the author from 2007 to 2017 in KwaZulu-Natal, South Africa, whilst registered at the University of KwaZulu-Natal, Pietermaritzburg, College of Agriculture, Engineering and Science (Formally Faculty of Science and Agriculture) School of Life Sciences (Formally School of Biological and Conservation Sciences), under the supervision of Professor Steven D. Johnson.

This dissertation, submitted in requirement for the degree of Doctor of Philosophy in the College of Agriculture, Engineering and Science, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not otherwise been submitted in any form for any diploma or degree to any tertiary institution or university. Where use has been made of the work of others, it is duly acknowledged in the text.

[redacted]. .

Ian Kiepiel
November 2019

I certify the above statement is correct.

. [redacted] . .

Professor Steven D. Johnson (supervisor)
November 2019

As the candidate's supervisor, I have approved this thesis for submission.

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DECLARATION 1 - PLAGIARISM

I, Ian Kiepiel, declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS THAT FORM PART AND/OR INCLUDE RESEARCH PRESENTED IN THIS THESIS

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Author contributions:

Ian Kiepiel and Steven D. Johnson conceived the paper. Ian Kiepiel collected and analysed the data, and wrote the paper. Steven D. Johnson contributed comments.

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Author contributions:

Ian Kiepiel and Steven D. Johnson conceived the paper. Ian Kiepiel collected and analysed the data, and wrote the paper. Steven D. Johnson contributed comments.

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LIST OF ABBREVIATIONS

a.s.l.	Above sea level
BW	Bushwillow
CAF	Central Analytical Facility, Stellenbosch University, South Africa
cm	centimetre
EAG	Electroantennogram
EAD	Electroantennographic detection
GC-EAD	Gas chromatography with electroantennographic detection
GG	Gibba Gorge
GSI	Gametophytic self-incompatibility
km	Kilometre
LD_{50}	Lethal dose
LSI	Late-acting self-incompatibility
m	metre
M	Molar concentration
MPNR	Mbona Private Nature Reserve
mm	Millimetre
OS	Ovarian sterility
OSI	Ovarian self-incompatibility
PC	Pondoland Centre of Endemism
PCR	Polymerase chain reaction
PER	Proboscis extension reflex
SI	Self-incompatibility
SSI	Sporophytic self-incompatibility
SSR	Simple sequence repeats or microsatellites
UNR	Umtamvuna Nature Reserve
UV	Ultraviolet
v	Volts

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CHAPTER 1

INTRODUCTION

MILIEU

Flowers exemplify the complexity and diversity of evolution and yet the myriad floral forms are each unified by adaptation involving the evolution of traits that improve mating success. The origin and significance of floral diversification has remained a central topic in biology following Darwin's treatise on natural selection (Darwin, 1859) and the application of this principle to floral evolution (Darwin, 1862a; Darwin, 1877). Following its initial public furore, Darwin's epiphany began to imbue the sciences with a newfound logic and the theory became the catalyst for a novel scientific paradigm. The recognition of adaptation through natural selection as the driving force behind speciation heralded the opening of a veritable Pandora's Box of evolutionary enquiry. This programme inevitably began to utilise the prodigious diversity of plant-pollinator interactions to provide model systems for the investigation of evolutionary biology. Now, the emergence of technological advancements are allowing for increasingly finer scale resolution of the factors underlying the basis of selection.

DARWIN'S "*ABOMINABLE*" MYSTERY AND POLLINATOR-DRIVEN DIVERSIFICATION

Early pollination studies in the Victorian era largely comprised of natural history monographs which lacked the insight of evolutionary theory (Sprengel, 1793; Müller, 1883; Knuth, 1906). Joseph Gottlieb Kölreuter (1761) and Christian Konrad Sprengel (1793) were arguably the first to put forward the notion that flowers function to attract insects (Baker, 1979). Insights from Sprengel's examination of floral form (Sprengel, 1793) were preludes to Darwin's thesis of floral adaptation stemming from selection for traits that enhance mating (Baker, 1979; Waser, 2006). Charles Darwin was also the first to propose that angiosperm diversification was a product of floral trait selection imposed by an assortment of pollination vectors (Darwin, 1859, 1862b, 1877). In doing so, he provided a partial solution to his own characterization of the rapid radiation of the flowering plants as an "*abominable mystery*". Darwin provided a novel adaptationist framework for the study of floral biology (Harder and Johnson, 2009), particularly with his work on orchid pollination (Darwin, 1862b, 1877). Building on Darwin's hypothesis, studies began to emerge suggesting that the cognition, morphology and sensory perception of pollinators could explain many existing floral traits (Baker, 1963; Grant and Grant, 1965; Stebbins, 1970). Darwin's theory of pollinator-driven diversification has gained steady support from studies demonstrating that pollinator shifts have occurred numerous times in various angiosperms lineages (Grant, 1949; Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006; Van der Niet and Johnson, 2012; Ollerton et al., 2019). Determining the origins of angiosperm diversity has and continues to be elemental to evolutionary and floral biology (Johnson, 2006).

The concept of pollinator-driven diversification suggests that shifts in primary pollinators and pollinator assemblages are strongly associated with adaptive floral trait modification and are considered a key mechanism of angiosperm diversification (Johnson, 2006; Harder and Johnson, 2009). Interest in pollinator-driven diversification only began to flourish in the 1960's with the advent of biosystematics and ecology, which was followed in the latter part of the past century by progress in comparative biology and the use of phylogenetic tools (Johnson, 2010). There is now a convincing body of evidence which ascribes the diverse complexity of floral form and function to adaptations for pollination (Stebbins, 1970; Dodd, Silvertown, and Chase, 1999; Bernardello et al., 2001; Johnson, 2006, 2010). Grant and Grant (1965) and Stebbins (1970) are credited with the initial development of this theoretical model of pollinator-driven diversification, now known as the Grant-Stebbins model (Johnson, 2006). This paradigm suggests that floral diversification is the outcome of recurring evolutionary shifts among various pollinating agents (Grant, 1949; Grant and Grant, 1965; Stebbins, 1970). Using support from their work on divergent pollination systems in the Polemoniaceae, Grant and Grant (1965) coined the term "*pollination climate*" in recognition of the fact that pollinator abundance varies across a species geographical range. They suggested that geographical discrepancies in pollinator availability would lead to differential vector utilization and selection, with consequent floral trait adaptation directed towards locally abundant pollinator assemblages (Grant and Grant, 1965). They argued that pollinator shifts would facilitate speciation through adaptive specialization, with the ensuing morphological divergence ultimately contributing to reproductive isolation (Grant and Grant, 1965). Ledyard Stebbins (1970) expounded on this, highlighting five key principles directing pollinator-driven adaptive radiation: (1) "*the most effective pollinator principle*", (2) "*the significance of character syndromes*", (3) "*selection along the lines of least resistance*", (4) "*transfer of function via an intermediate stage of double function*" and (5) "*reversals of evolutionary trends*".

Albeit alluring in its rationale, the Grant-Stebbins model has yet to engender a wholly convincing body of corroborative evidence linking shifts according to the "*pollination climate*" with broader patterns of pollinator-driven diversification (Johnson, 2010), an occurrence Stebbins (1970) ascribed to the predominance of microevolutionary studies rather than macroevolutionary comparative approaches (a trend that has been somewhat reversed today). Although many studies have used an evolutionary framework for the study of pollination interactions (Grant, 1949; Schemske and Bradshaw, 1999), few have covered both macroevolutionary processes as well as microevolutionary variations associated with pollinator-driven phenotypic selection (Campbell et al., 1991; Bradshaw and Schemske, 2003). Research into floral trait selection has thus frequently been enumerated at single sites, with limited studies investigating trait selection across multiple pollination ecotypes in different geographical areas (Fenster and Dudash, 2001; Moeller, 2005; Nattero and Cocucci, 2007) or attempting to obtain quantitative measures of pollinator assemblages amongst sister taxa (Johnson, 2006; Rey et al., 2006). The fact that so few studies have entailed the examination of selection at the level of the phenotype over various sites (Johnson and Steiner, 1997; Totland, 2001; Herrera et al., 2002) has been suggested by Johnson (2006) to be a shortcoming of pollination research, and one which limits our ability to attribute variation to local adaptation.

There is some compelling evidence in support of the Grant-Stebbins model (see Johnson, 2006), which includes, the relationship between certain pollinators and pronounced floral genotypes (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003), the prominence of selection forces imposed by pollinators on floral traits (Herrera, 1993; Campbell, Waser, and Meléndez-Ackerman, 1997; Gervasi and Schiestl, 2017), as well as studies demonstrating that reproductive isolation in sympatric species is associated with diverse pollinator utilization (Stebbins, 1970; Grant, 1981; Ellis and Johnson, 1999; Ramsey, Bradshaw, and Schemske, 2003; Dell'Olivo et al., 2011). Substantiation of the model also comes from phylogenetic evidence of recurring evolutionary shifts between diverse pollination vectors in numerous angiosperm lines (Johnson, Linder, and Steiner, 1998; Beardsley, Yen, and Olmstead, 2003; Kay et al., 2005; Whittall and Hodges, 2007; Serrano-Serrano et al., 2017; Smith and Kriebel, 2018; Ollerton et al., 2019). Another line of evidence, implicates the relative synchronized radiation of flowering plants and allied insects (Eriksson and Bremer, 1992). The Grant-Stebbins model (Johnson, 2006) has also been categorically been linked to intraspecific variation of floral traits in a number of families with diverse pollination systems (Grant and Grant, 1965; Miller, 1981; Robertson and Wyatt, 1990; Goldblatt and Manning, 1996; Johnson, 1997; Johnson and Steiner, 1997; Martén-Rodríguez et al., 2011; Peter and Johnson, 2013; Sun, Gross, and Schiestl, 2013; Gervasi and Schiestl, 2017).

However, on the contrary, some studies have failed at attempts to link floral trait diversification to geographical variation of pollinator assemblages (Miller, 1981; Herrera et al., 2002; Scobell and Scott, 2002). Such examples may indicate evolutionary processes besides pollinator shifts in driving floral divergence, or alternatively, may reflect difficulties in relating shifts to current environments owing to environmental changes since the time of divergence. A handful of studies have identified floral trait variation in lineages which utilize the same pollinating agents (Macior, 1982; Steiner, 1989; Schemske and Bierzychudek, 2001; Pauw, 2006; Ellis and Johnson, 2009). In some of these taxa, diversification is thought to be a response to pollen limitation originating through competition among several species sharing the same pollinators, and involves trait modifications effecting pollen placement (Macior, 1982; Johnson and Bond 1994). Although speciation driven by pollinators has been predicted to be the most prevalent and convincing examples of ecological speciation (Van der Niet, Peakall, and Johnson, 2014), since the radiation of a number of specialized angiosperm clades has taken place with fixed pollination dynamics (De Luca and Vallejo-Marin, 2013; Davis et al., 2014), clarification is still needed in determining how pollinator shifts explain diversity on a broader scale (Van der Niet, Peakall, and Johnson, 2014).

Pollinator-driven diversification is of course only one of several plausible modes of angiosperm evolution. Diversification need not necessarily be associated with pollinators alone (Strauss and Whittall, 2006), and there are well documented cases of speciation driven by abiotic factors (Baldwin, 1997) and a combination of abiotic and biotic factors (Lagomarsino et al., 2016). Selection may also be imposed by herbivores (Theis and Adler, 2012; Ågren et al., 2013; Jogesh et al., 2017) and pathogens (Shykoff, Bucheli, and Kaltz, 1997). Herbivores may play significant roles in plant diversification by influencing interactions between pollinators (Ehrlich and Raven, 1964; Janz, 2011; Althoff, Segraves, and Johnson, 2014; Johnson, Campbell, and Barrett, 2015; Marquis et al., 2016), or by imposing selection on various floral traits such as morphology (Galen and Cuba, 2001; Jogesh et al., 2017), odour (Gross, Sun, and Schiestl, 2016), colour (Irwin et al., 2003; Frey, 2004; Carlson and Holsinger, 2012), flowering phenology (Brody, 1997), and even mating systems (Kariyat et al., 2013; Carr and Eubanks, 2014). There has been a resurgent interest in floral diversification driven by herbivory (Johnson, Campbell, and Barrett, 2015; Marquis et al., 2016) and growing evidence in support of this (Galen, 1999; Herrera, 2000; Cariveau et al., 2004; Carlson and Holsinger, 2012; Sun, Armbruster, and Huang, 2016; Jogesh et al., 2017). Regardless of the particulars of variation speciation models, a consensus appears to have been reached that natural selection is the predominant basis of speciation (Coyne and Orr, 2004). This has become known as ecological speciation (Nosil, 2012). Ecological speciation provides a valuable contextual application in connecting macroevolutionary studies on speciation with microevolutionary adaptation research (Nosil, 2012). This offers investigable predictions, which are applicable to each tier in the cascade of speciation (Van der Niet, Peakall, and Johnson, 2014). Recent studies have emerged indicating real-time pollinator-driven evolution of divergent floral traits (Gervasi and Schiestl, 2017). Such novel experimental approaches have tested the Grant-Stebbins model and quantified pollinator driven selection, revealing that different pollinators may drive adaptive floral evolution (Gervasi and Schiestl, 2017). Increasingly, such studies are finding new relevance as indicators of changing pollinator assemblages associated with habitat loss and climate change (Biesmeijer et al., 2006; Hegland et al., 2009; Gervasi and Schiestl, 2017).

THE PARADIGM OF THE POLLINATION SYNDROME

Frederico Delpino was the first to propose the convergence of floral traits in taxonomically diverse species which share pollinators (Delpino, 1867). This theory was expounded upon by Kunth (Knuth, 1906) and subsequently indorsed by numerous others, and was later termed pollination syndromes (Vogel, 1954; Baker, 1963; Grant and Grant, 1965; Stebbins, 1970; Faegri and van der Pijl, 1979). Fundamentally, the concept suggests that unrelated taxa often display convergent suites of floral traits which reflect adaptations to the particular mode of pollination and, in the case of biotic vectors, the ethology of the relevant pollinator (Stebbins, 1970; Faegri and van der Pijl, 1979; Fenster et al., 2004). The pollination or floral syndrome concept represents an attempt to identify adaptations from general patterns of convergent evolution. As such, far from being an obdurate system of classification, it embodies an agenda which has greatly assisted research into pollination biology (Proctor, Yeo, and Lack, 1996). The syndrome concept implies that pollination systems are specialized, at least at the level of functional groups, be they abiotic (e.g. water, wind) or biotic (e.g. bat, bird, butterfly, etc.). A substantial part of the debate around syndromes relates to our understanding of how much specialization exists in pollination systems (Herrera, 1996; Ollerton, 1996; Waser et al., 1996; Johnson and Steiner, 2000; Fenster et al., 2004). In light of evidence that multiple pollinators visit a large number of plant species, it was proposed that generalization rather than specialization is more prevalent in pollination systems (Waser et al., 1996). This roused an intense debate, stimulating research on the utility of the concept (Fenster et al., 2004; Ollerton et al., 2009; Rosas-Guerrero et al., 2014; Ashworth et al., 2015; Johnson and Wester, 2017). Among the criticisms, are that the concept fails to take into account dynamic temporal fluctuations in the pollination environment and treats pollination mutualisms as strictly specialist or generalist, rather than as a dynamic continuum. The issue is also complicated by the fact that an array of disparate methods are utilized for quantifying specificity in pollination systems (Johnson and Steiner, 2000).

There can be no doubt that a large proportion of angiosperms do in fact possess generalised pollination systems, at least in the sense of being pollinated by many different species (Ollerton, 1996; Waser et al., 1996). Generalized systems do not lend themselves to definitive syndrome categorization and this has rightly led to the questioning of the validity and utility of the syndrome paradigm (Waser et al., 1996). Whilst compelling evidence suggests that at many levels, selection for floral traits that enhance mating success (sexual selection), has been key to the radiation and diversity of the angiosperms (Grant and Grant, 1965; Stebbins, 1970; Johnson and Bond, 1994; Goldblatt and Manning, 2006), classification of suites of traits into a syndromes representing groups of pollen vectors provides varying degrees of clarity. However, several researchers have pointed out that there is considerable evidence for pollination system specialization at the level of functional pollinator assemblages (Johnson and Steiner, 2000; Fenster et al., 2004; Johnson and Wester, 2017). Robust validation for syndrome association within particular pollinator clusters is still emerging, adding credence to both the pollination syndrome paradigm as well as pollinator-mediated floral trait selection (Johnson and Wester, 2017).

Specialized pollination systems often lend themselves to predictions of formerly unknown pollinators based on assemblages of floral features (Johnson, Pauw, and Midgley, 2001; Hargreaves, Johnson, and Nol, 2004; Pauw, 2006). However, floral traits can be incredibly diverse even in highly specialized systems (Johnson and Steiner, 2000; Fenster et al., 2004) and the concept obviously has little predictive use for plants that have generalized pollination systems (Hingston and Mcquillan, 2000; Ollerton et al., 2003; Wilson et al., 2004; Zhang et al., 2005; Valdivia and Niemeyer, 2006; Ollerton et al., 2009; although see Rosas-Guerrero et al., 2014). The utility of the syndrome concept is likely to be geographically variable, with its predictive value correlated with the degree of pollination specialization in each region (Johnson and Steiner, 2003; Rosas-Guerrero et al., 2014; Ashworth et al., 2015), as has been shown to be the case for the biodiversity rich specialized pollination systems of South Africa (Johnson and Wester, 2017). There many instances, particularly in southern Africa, where convergent suites of floral traits have been shown to strongly confirm to floral adaptation to pollinators (Johnson and Bond, 1994; Johnson and Steiner, 1997; Johnson and Wester, 2017). The widespread pollination system specialization in the southern African floral illustrates geographical variability in the continuum (Johnson and Steiner, 2000; Johnson and Steiner, 2003; Goldblatt and Manning, 2006; Van der Niet and Johnson, 2009; Johnson and Wester, 2017), and offers a contrast to the more generalized systems of many temperate northern hemisphere flora (Waser et al., 1996; Johnson and Steiner, 2000; Johnson and Steiner, 2003; Ollerton, Johnson, and Hingston, 2006). It is pertinent to note, as pointed out by

Waster et al., (1996), that the pollination syndrome paradigm has been used without recognizing that there is a continuum of specialization in pollination systems. In the past decade, the view has emerged that pollination syndromes are not universal in the sense that they are unlikely to universally account for the diversity of floral forms (Ollerton et al., 2009; Ollerton et al., 2015; Smith and Kriebel, 2018).

Perhaps more than any other function, the continued use of the concept designates its utility in pinpointing traits on which selection acts upon (Wilson et al., 2004; Pauw, 2006; Whittall and Hodges, 2007). Although still in its infancy, the application of molecular markers will likely provide much needed insight into the relationship between floral adaption and functional pollinator groups on a genetic level (Galliot, Stuurman, and Kuhlemeier, 2006; Hermann and Kuhlemeir, 2011). The examination of specific floral traits to determine the consequences of polymorphism on pollinators will greatly aid in understanding the contribution of specific genes to pollination syndrome adaption (Galliot, Stuurman, and Kuhlemeier, 2006). The use of high-throughput genome sequencing promises to greatly expedite such inquests (Hermann and Kuhlemeir, 2011).

FLORAL ADVERTISING SIGNALS AND MORPHOLOGY

A flower represents a historical pattern of pollination, the anthology of a plants sexual relations; articulating multifaceted sensory information and acting as a mnemonic to pollinators. For well over two hundred years, biologists have tried to make sense of floral forms, colours and scents (Sprengel, 1793). Advertising signals are usually considered as cues that aid pollinators in determining the quality as well as quantity of floral rewards available (Cresswell and Galen, 1991; Sutherland and Vickery, 1993; Makino and Sakai, 2007), but more properly that can be considered as traits by which plants exploit animal perception, cognition and behaviour for the purposes of pollination (Schiestl and Johnson, 2013). Floral architecture may be considered simply as the suite of morphological adaptations to pollination best tailored to the local pollinator mosaic (Grant and Grant, 1965; Stebbins, 1970). Floral traits such as fragrance, colour, size and shape provide vital signals to pollinators and act as cues to distinguish species and locate floral rewards (Chittka and Raine, 2006). Plants employing abiotic pollination vectors need not advertise and instead concentrate their reproductive efforts into maximising the efficiency of gamete production and transfer, seed development, and dispersal.

Plants using biotic agents for pollination need to advertise to potential vectors, and use a variety of signals in order to draw their attention. Floral advertisements intended to entice pollinators towards reproductive structures take the form of olfactory (scent) as well as visual stimuli such as colour and shape. Frequently, pollinators depend on olfactory and visual signals to locate essential floral resources such as food (Dobson, 1994). Conditioning due to associations with floral rewards, as well as initial attraction due to pre-existing bias or experience with other plants, ensures pollinator constancy and promotes successful pollination (Schiestl and Johnson, 2013). Floral constancy or flower fidelity implies that pollinators are able distinguish between the flowers of one species from those of another and remember those differences as well as the rewards they may offer (Dafni, 1992). Remarkably, it is precisely this ability of an animal to learn various environmental cues and interpret them in a meaningful dynamically adaptable manner which, comparative physiologists use as a measure of intelligence. Some plants abuse pollinator constancy through the use of mimicry, thus deceiving the pollinator for gain without pay (Dafni, 1984; Schaefer and Ruxton, 2009). The majority of plants however offer recompense to animals for their pollination services (Simpson and Neff, 1983). Floral advertisements often change during the life of a flower and sometimes abruptly, after pollination (Lamont, 1985). After a flower is spent, typically its petals wither or close, inevitably to abscise. During this senescence, floral colours may change as nectar ceases to be produced, and fragrances become fainter, providing valuable postpollination communication to pollinators (Theis and Raguso, 2005).

Evidence suggests that pollinator sensory preferences effect the evolution of floral advertising signals (Vereecken and Schiestl, 2008; Schiestl and Johnson, 2013). Floral advertising significantly exploits and influences the behaviour of pollinators which in turn has important consequences for plant fitness, influencing conditions such as geitonogamy, inbreeding, and pollen discounting (Chittka, Thomson, and Waser, 1999). Advertising signals that encourage proficient learning and behaviour, and promote effectual pollen transfer, are of clearly great advantage to plant fitness (Dobson and Bergström, 2000). The ideal pollinator will thus be one which visits numerous conspecific plants in a population, ensuring maximum cross-pollination by avoiding self-pollination and curtailing pollen and ovule discounting (Waser and Campbell, 2004). From a pollinator’s perspective, it is critical to learn and retain the memory of floral traits associated with food so that foraging efficiency can be optimised by lessening the energetic demands of searching for reward offering plants (Waser, 1986; Goulson and Cory, 1993; Lewis, 1993).

Floral scent is the most far-reaching (in terms of distance) of all floral advertising signals. Production of scent is an expensive metabolic affair (Vogel, 1983) and yet flowers are frequently perfumed – a scenario which has both positive as well as negative ramifications for plants using animal pollinators (Raguso, 2001). Floral scent may for example attract the unwanted presence of herbivores which, like pollinators use odours in food location (Baldwin, 1997; Galen and Butchart, 2003). Scents often convey exceptionally intricate signals to animals and are conspicuous amongst floral advertisements because of the complexity and variation of chemical components produced both within and amongst taxa (Raguso, 2001; Dobson, 2006). Floral scent is composed of a wide variety of organic compounds, sometimes consisting of up to several hundred diverse volatile compounds (Knudsen, Tollsten, and Bergström, 1993). Although floral scent is diverse, it appears to be dominated by odours consisting of benzenoids, terpenoids and aliphatic compounds (Knudsen, Tollsten, and Bergström, 1993). Yet, a just single odour compound can have manifold functions in chemical signalling (Blum, 1996).

Scent is particularly important in attracting pollinators from large distances (Metcalf and Metcalf, 1992; Raguso, 2008) and is a highly significant floral advertisement to pollinators of nocturnal blooms, the animals of which cannot rely exclusively on visual signals to find resources (Raguso et al., 2003; Hoballah et al., 2005). Scent has been found to endorse reliability and effectiveness of pollinators and may be habitually mutually advantageous to both plant and animal vectors alike (Dornhaus and Chittka, 1999). Some flowers produce nectar which is scented (Raguso, 2004) and scented pollen is produced by a number of species (Dobson, Bergström, and Groth, 1990; Bergström, Dobson, and Groth, 1995; Dobson, Groth, and Bergstrom, 1996; Dobson and Bergström, 2000). Spatial patterns of fragrance may aid pollinator orientation by functioning as fragranced nectar guides (Bergström, Dobson, and Groth, 1995; Lawson, Whitney, and Rands, 2017). Floral scent can also be subject to selection and can encourage specificity in pollinators (Groth, Bergstrom, and Pellmyr, 1987; Whitten and Williams, 1992; Mant, Peakall, and Schiestl, 2005; Schiestl, Huber, and Gomez, 2011). Complex chemistry and variety of scent is thought to arbitrate various floral roles, for instance promoting floral constancy and pollinator attraction (Dobson, 1994; Raguso, 2008). Floral scent can also be correlated with adaptations for food, mate and brood-site location that are found in certain groups of pollinators (Jurgens, Witt, and Gottsberger, 2003; Huber et al., 2005). Although there are numerous studies which have undertaken detailed analysis of the chemical make-up of scent in plants (Andersson et al., 2002; Dobson, 2006; Knudsen and Gershenzon, 2006), we have much to learn of the selection forces governing the evolution of floral scent variation and composition (Schiestl, 2010). Elucidating the role of floral scent in the association between plants and their pollinators has in the past been ascertained through broad comparative studies of floral convergence (Knudsen and Tollsten, 1995; Reznick and Ricklefs, 2009; Jűrgens et al., 2013) and experimental studies of how scent increases floral appeal to pollinators (Pichersky and Gang, 2000).

There are an increasing number of studies that look at synergy between volatile and visual cues (Ômura, Honda, and Hayashi, 1999; Raguso and Willis, 2002; Ômura and Honda, 2005; Raguso and Willis, 2005; Hirota et al., 2019). Colour is thought to be the most ubiquitous of all the floral signals (Menzel and Shmida, 1993), but primary advertising signals to pollinators are often a combination of scent and visual cues (Raguso, 2004; Ômura and Honda, 2005). A pollinator's floral choice relies on a number of signalling cues, but numerous studies have shown that for certain pollinators, colour represents the most important basis for foraging choices (Dafni and Bernhardt, 1990; Ômura and Honda, 2005; Dötterl et al., 2014). Colour manifests through the presence of pigments which absorb components of the visible light spectrum, including UV (ultraviolet), with the result that light of a particular wavelength which is not absorbed is reflected back, the perception of which also depends on the visual systems of the individual animal in question (Grotewold, 2006). Colour perception in animals is therefore determined by the visual receptors and the neural opponency pathways inherent in decoding spectral information (Chittka and Menzel, 1992; Kelber, Vorobyev, and Osorio, 2003; Kelber and Osorio, 2010). Colouration plays an integral function in attracting pollinators to flowers and affects both the preference of pollinators (Bradshaw and Schemske, 2003) as well as pollinator constancy with respect to various floral forms (Hill, Wells, and Wells, 1997; Keasar et al., 1997). Not surprisingly, there exists a complex synergy between various floral signals such that pollinator constancy can also be affected by other nuances such as handling time (Sanderson et al., 2006) and scent (Andersson, 2003).

Floral nectar guides are simple coloured patterns (Sprengel, 1793) which are found throughout numerous families (Weiss, 1995). They are important advertising signals, assisting pollinators in locating rewards (Kevan, 1972; Lunau, 1992) and promoting the efficiency of pollen transfer (Casper and La Pine, 1984). Nectar guides effect pollinator preferences and at close-range distances many insects find flowers with these patterns more attractive than flowers without them (Manning, 1956; Waser and Price, 1985; Dafni and Giurfa, 1999; Dinkel and Lunau, 2001). Although their presence may increase the variety of pollinators visiting a species (Ollerton et al., 2007), of great significance is the fact that they have been shown to improve the rate of pollen transfer and reduce pollinator detection and handling times of floral rewards (Penny, 1983; Waser and Price, 1983; Leonard and Papaj, 2011). Certain plants are able to change the colours of nectar guides as floral rewards are depleted (Casper and La Pine, 1984; Delph and Lively, 1989; Zang et al., 2017), facilitating outcrossing by informing pollinators of older, rewardless flowers, and assisting in the location of freshly opened flowers (Casper and La Pine, 1984). The potential increase in pollen transfer efficiency is an important fitness advantage to nectar guides (Medel, Botto-Mahan, and Kalin-Arroyo, 2003; Schaefer, Schaefer, and Levey, 2004; Leonard and Papaj, 2011; Hansen, Van der Niet, and Johnson, 2012).

Floral colour is not only a mechanism of pollinator attraction, but can also function to enhance other aspects of plant fitness such as drought tolerance (Warren and MacKenzie, 2001), the amelioration of herbivory (Simms and Bucher, 1996), or staving off damage from pathogens (Frey, 2004). Plant pigments consist of three classes of chemicals - betalains, carotenoids and flavonoids. Betalains are found only in the Caryophyllales (producing for example, the characteristic red pigment found in beetroot), whilst carotenoids are ubiquitous lipid-soluble plastid-oriented pigments responsible for orange and yellow colours (Whitney and Glover, 2007). Flavonoids are water soluble vacuole-oriented pigments, composed of flavones and flavonols (cream colours), aurones and chalcones (yellow and orange colours), and the widespread anthocyanins, which give rise to shades of blue, red, pink and purple (Whitney and Glover, 2007). Biochemistry involving the synthesis of colour pigments is relatively well understood in plants (Grotewold, 2006). In most instances, patterns of floral colour are regulated by the expression of genes which encode various transcription factors responsible for controlling pigment biosynthesis (Mol, Grotewold, and Koes, 1998). Studies explicating the genetic basis of floral colour have undertaken the mapping of pigment production in species with respect to their associated pollinators (Bradshaw and Schemske, 2003; Whittall et al., 2006; Hoballah et al., 2007). It is now clear that since genes control floral pigmentation, which is an important floral advertising cue, changes in genes that mediate flower colour can quickly cause shifts in pollinators (Bradshaw and Schemske, 2003; Hoballah et al., 2007).

Flowers vary in symmetry (asymmetrical, bilaterally or radially symmetrical) and floral forms can be of various shapes such as bowl, brush, classic, cup, flag, horizontal and trumpet type flowers

(Faegri and van der Pijl, 1979; Dafni and Kevan, 1997). Pollinators often use the shape of a flower as a signalling cue and floral shape represents a morphological trait which can have a significant impact on plant fitness due to its interaction with pollinator morphology (Herrera, 1989). In comparison to colour vision in pollinators, there is far less known about the mechanisms of perception involved in pollinator shape recognition (Dafni, Lehrer, and Keyan, 1997; Campos, Bradshaw, and Daniel, 2015). Shape directly influences pollinator efficiency (Campbell, Waser, and Price, 1996; La Rosa and Conner, 2017) as various floral forms may present mechanical barriers to certain visitors, allowing floral access to only those pollinators with the correct morphological attributes (Nilsson, 1988). Floral shape may represent a mechanical isolating barrier driving evolutionary diversification between plants utilizing different pollinator groups (Castellanos, Wilson, and Thomson, 2004; Muchhala, 2007; Smith and Kriebel, 2018). Surprisingly few studies have empirically demonstrated at the macroevolutionary scale the relationship between floral shape and the pollination system, but convincing evidence is emerging which suggests that modification of corolla shape is frequently associated with pollinator shifts (Smith and Kriebel, 2018).

Additional selective pressures on morphological appearance include, the prevention of self-pollination and the promotion of cross-pollination (Proctor, Yeo, and Lack, 1996) as well as the protection of nectar and pollen from dishonest animals or environmental exposure (Armbruster, 1996). Contrasting outlines between the corolla and the floral background also play a significant role in visual orientation for pollinators which can impose selection on floral shape through their ability to find rewards (Waser and Price, 1985; Lunau et al., 2006; Koshitaka, Arikawa, and Kinoshita, 2011). In some cases, pollinators show preferences towards corollas which are symmetrical rather than those of irregular appearance (Kelber, 1997; Møller and Sorci, 1998). Floral shape as well as the size of a flower appear to work harmoniously in pollinator attraction (Dafni, Lehrer, and Keyan, 1997). Floral size and the overall morphology of a flower are strongly influenced by the needs of a species to attract suitable pollinators (Faegri and van der Pijl, 1979; Armbruster, 1996; Conner and Rush, 1996). As well as floral size, the size of the inflorescence can often affect pollinator visitation (Bell, 1985; Herrera, 1993). The consequences of floral display size with respect to pollination is one of the most studied attributes of plant inflorescences (Harder and Barrett, 1996; Harder et al., 2001). Generally, plants which are large, produce more flowers, which in turn attract more visitors to their floral displays than do plants of smaller stature (Schaffer and Schaffer, 1979; Klinkhamer, de Jong, and Debruyn, 1989; Klinkhamer and de Jong, 1990). Similarly, plants with big inflorescences generally draw more visitors than individuals with smaller bouquets (Cruzan, Neal, and Willson, 1988; Schmid-Hempel and Speiser, 1988; Thomson, 1988; Pleasants and Zimmerman, 1990). Likewise, at the population level, a large group of conspecific plants will result in higher visitation rates to individuals in the population compared to those individuals which are dispersed or isolated (Silander, 1978; Klinkhamer, de Jong, and Wesselingh, 1991). Not only do larger flowers encourage greater visitation rates compared to smaller flowers in a population (Bell, 1985; Ashman and Stanton, 1991), but compared to smaller flowers, they also promote superior pollination intensity or deposition of conspecific pollen on stigmas (Galen and Newport, 1987). Insects are generally attracted to larger rather than smaller flowers (Dafni, 1997; Spaethe, Tautz, and Chittka, 2001) and it is thought that larger flower sizes are preferred and have been evolutionarily favoured because of the inadequate resolving ability of the insect eye (Chittka and Raine, 2006). However, larger flowers may be favoured simply due to the strong correlation between the size of a flower and the volume of nectar available (Herrera, 1985; Dafni, 1991).

PHYLOGENETIC PERSPECTIVES ON POLLINATOR SHIFTS

There remains little doubt that pollinators have influenced the evolutionary diversification of angiosperms (Grant and Grant, 1965; Stebbins, 1970; Faegri and van der Pijl, 1979), but the actual mechanisms of diversification remain contested. One of the most compelling mechanisms is the Grant-Stebbins model of pollinator shifts, which has received strong support from both micro- and macroevolutionary studies (Kay et al., 2005; Johnson, 2006; Pauw, 2006; Smith, Ane, and Baum, 2008; Reynolds, Dudash, and Fenster, 2010; Van der Niet and Johnson, 2012; Forest et al., 2014). Microevolutionary studies directed at identifying the population-level processes of diversification on divergence in single species are, and will remain, extremely relevant to revealing the role that pollinators play in floral diversification (Harder and Johnson, 2009). However, such studies are limited in their ability to predict the extent to which pollinator-driven diversification shapes broader macroevolutionary patterns (Johnson, 2010).

In recent years there has been a renewed interest in macroevolutionary patterns of diversification (Ricklefs, 2004, 2006; Kreft and Jetz, 2007; Fiz-Palacios et al., 2011). The use of phylogenetic methods in the study of floral diversification, pollinator shifts and plant evolution is comparatively recent, yet offers biologists important tools for understanding plant diversity (Weller and Sakai, 1999). Macroevolutionary diversity is thought to arise through selection of traits at the level of the population (Johnson, 2006, 2010). Adaptations spread among adjacent populations can become fixed at the species level by a combination of reproductive isolation and extinctions of intermediate forms, which accentuate discontinuities among contemporary species (Reznick and Ricklefs, 2009). Phylogenetic evidence strongly supports the radiation of the angiosperms through diversification driven by shifts among various pollinators (Van der Niet and Johnson, 2012). This evidence suggests that pollinator shifts have often taken place in numerous plant lineages around the world (Givnish and Sytsma, 1997; Weller and Sakai, 1999; Breitkopf et al., 2015). However, a recent reconstruction of an extensive phylogeny from the Gesneriaceae, suggests that pollinator shifts were not responsible for diversification in the family, but rather, diversification is thought to have occurred within lineages of hummingbird-pollination and without pollinator shifts between functional groups (Serrano-Serrano et al., 2017). Significantly, phylogenetic trees constructed from molecular sequencing data are also capable of calculating approximate rates of speciation and the timing of divergence events (Hey, 1992; Nee, 2007).

As more and more phylogenies for various levels of plant taxa are produced, additional information regarding evolutionary shifts in pollination systems can be inferred (Sapir and Armbruster, 2010). The combination of molecular systematics and cladistic methods has highlighted the efficacy of coalescing ecology with systematics and has significantly boosted phylogenetic research (Barrett, 1995; Barrett, Harder, and Worley, 1996). Information from comprehensive phylogenies may be effectively combined with ecological as well as pollination system data in order to determine patterns of evolutionary diversification (Smith, Ane, and Baum, 2008; Ollerton et al., 2019). The development of phylogenetic trees provides biologists with tools for examining the evolutionary processes responsible for angiosperm diversity which, when combined with ecological and geographical information of taxa, can yield significant insight into the origin of speciation within or between clades (Barraclough, Vogler, and Harvey, 1998). However, the reliance on molecular approaches to understand the evolution of flowers (Glover et al., 2015; Specht and Howarth, 2015) may have its limitations (De Craene, 2018). Originally, as Weller and Sakai (1999) pointed out, the ability to make inferences from phylogenetic trees was constrained by the shortage of well-supported phylogenies, but increasingly it is the lack of natural history data on pollination systems that limits our ability to make inference about drivers of floral diversification, even when phylogenetic trees are available.

PLANT BREEDING SYSTEMS: UNDERSTANDING FACTORS INFLUENCING MATING

Understanding the reproductive biology of plants is essential for agriculture, biodiversity conservation, horticulture, invasive species control and the development of biotechnology (Barrett, 2010). Plant breeding systems encompass the morphological organization of reproductive structures and their inherent mechanisms of reproduction (Sage, Husband, and Routley, 2005). Plant mating systems in turn represent the level of outcrossing and are responsible for the genetic makeup of offspring (Sage, Husband, and Routley, 2005). Up until the last century, the majority of research into plant breeding and mating systems was focussed on northern hemisphere species - a reflection on the historical centres of learning. The gaps in the literature in themselves provide ample opportunities for pioneering research. Much like floral form, arguably the single unifying aspect of plant mating systems is the fundamental nature of gene transmission (Barrett, Baker, and Jesson, 2000). It has long been recognised that natural selection through working on the mode of gene transmission and the affiliated mating system, has governed floral evolution and the development of breeding systems in angiosperms (Barrett, Baker, and Jesson, 2000). The remarkable diversity of mating systems is thought to be the outcome of evolutionary interactions between reproductive traits and the ecology of plant populations (Barrett and Harder, 1996; Barrett, Baker, and Jesson, 2000; Barrett, Cole, and Herrera, 2004). Evolutionary diversity of plant mating systems ensures optimal reproduction given the potentially unstable and varied nature of ecological conditions (Barrett, Baker, and Jesson, 2000). This incredible diversity is often seen as the driving force for research into plant breeding systems (Uyenoyama, Holsinger, and Waller, 1993; Holsinger, 1996). Mating is fundamental to microevolution and is crucial to evolutionary biology

(Karron et al., 2011), and in the midst of anthropogeniclly fuelled habitat fragmentation and climate change, mating system research is progressively more important to conservation management (Coates, Sampson, and Yates, 2007; Eckert et al., 2010).

Although angiosperm mating strategies are said to be remarkably adaptable (Barrett, 2003), there appears to be a strong correlation between the mating systems of closely related taxa and their ecological niche (Barrett and Eckert, 1990). Variations in angiosperm mating systems are thought to be a consequence of the particular biology of plants and their adaptive responses to the diverse environments in which they inhabit (Barrett, 1998). Characteristics such as hermaphroditism, numerous reproductive structures, sessile modular growth, closed carpels and extraordinarily diverse life histories, have been identified as traits significantly influencing angiosperm diversity (Barrett and Harder, 1996; Barrett, 1998; Barrett, 2003). Since the majority of plants are hermaphroditic the opportunity exists for selfing, hence inquiry into plant mating systems has largely taken the form of comparative analysis of outcrossing and selfing rates (Lloyd and Schoen, 1992; Goodwillie, Kalisz, and Eckert, 2005). A limitation to this approach is that plants can outcross with a diverse variety of sources, which can differ considerably (Pannell and Labouche, 2013).

In order to fully comprehend the enormity of sexual multiplicity in the angiosperms, care must be taken to appreciate why such variations have evolved, as well as in resolving the means of reproduction at molecular, developmental and physiological levels (Barrett, 1998; Barrett and Harder, 2017). Although research into mating and pollination systems are interrelated, historically there has been surprisingly little intersect between the two disciplines (Fenster and Martén-Rodŕiguez, 2007). Latterly, more emphasis has been placed on conducting research into both mating and pollination systems simultaneously (Barrett and Harder, 1996; Holsinger, 1996; Barrett, 2003; Fenster and Martén-Rodŕiguez, 2007; Krauss et al., 2017). There is a growing appreciation of pollinator contributions to mating system selection through geitonogamous selfing (Lloyd, 1992; Harder and Barrett, 1995), pollen limitation (Morgan and Wilson, 2005), as well as pollen and ovule discounting (Harder and Wilson, 1998; Porcher and Lande, 2005; Johnston et al., 2009). Until fairly recently, studies on plant mating focused on theoretical models and population genetics, with little or no attention given to the ecological interactions ultimately governing angiosperm mating (Barrett and Harder, 1996). Conversely, it has been suggested that pollination research has tended to be somewhat misguided in its overtly ecological orientation, with a paradoxical disregard for the mating patterns which in essence determine plant fitness (Barrett and Harder, 1996). The isolation between the two fields appears inconsistent, given that adaptive shifts in pollination systems ultimately affect the mating system and *vice versa*. In recent years, mating system research has covered more diverse ecological topics including, the evolutionary ramifications of clonality (Vallejo-Marín, Dorken, and Barrett, 2010), evolutionary interactions in herbivore defence (Johnson, Campbell, and Barrett, 2015), and mating ecology and its evolutionary implications (Barrett and Harder, 2017).

Lloyd (1979) was perhaps the first to lead the way into an understanding of how plant adaptation to pollinators can affect selection on the mating system. Both pollination and breeding systems determine the flow of genes via pollen and seed dispersal amongst conspecific populations (Levin and Kerster, 1974). Plant mating systems can therefore act as driving forces for speciation because they ultimately govern gene flow between populations. Reproductive assurance, the avoidance of inbreeding depression, effective pollen dispersal and optimal resource allocation of male and female sexes are considered the principal determinants, selecting for breeding systems and mating strategies in angiosperms (Lloyd, 1979; Charlesworth and Charlesworth, 1987; Uyenoyama, Holsinger, and Waller, 1993; Barrett, 1998; Barrett, Baker, and Jesson, 2000). More recently, it has been suggested that selection of mating systems may be influenced not only by rates of maternal outcrossing, but also by mating success at the individual level and the diversity of mates (Barrett and Harder, 2017). These selective mechanisms are manifest in various suites of floral adaptations (Barrett, 2003). Not only can there be found a great diversity between different families with respect to their breeding systems and associated floral adaptations, but so too can there be found considerable variation at interspecific and intraspecific levels (Barrett, Baker, and Jesson, 2000).

Mating systems at both the individual and population level, may significantly impact gene flow and genetic diversity (Barrett and Harder, 2017). Theoretical models of plant mating systems indicate that there are two evolutionary stable endpoints of plant mating, namely predominantly outcrossing or predominantly selfing (Barrett, 2002). This does not always hold true, and some empirical research shows that mixed-mating occurs in at least a third of all plant species (Vogler and Kalisz, 2001; Goodwillie, Kalisz, and Eckert, 2005). One shortcoming of these studies is the emphasis on species level mating system variation, rather than population level disparity (Vogler and Kalisz, 2001; Goodwillie, Kalisz, and Eckert, 2005; Moeller et al., 2017). Even within a species, there may be variation in outcrossing rates, and it is not uncommon for individuals in a population to range from complete selfing to complete outcrossing (Barrett and Husband, 1990; Whitehead et al., 2018). A recent survey entailing 30 years of plant mating system literature, comprising of 105 species from 741 populations, indicates that mixed mating is common and that 63% of the species surveyed presented mixed mating systems in at least one population (Whitehead et al., 2018). Generally, self-compatible taxa have highly variable outcrossing rates which appear to be predominantly governed by prevailing ecological states (such as pollen limitation) and the fitness costs of selfing (Barrett, 2002). Molecular markers have become valuable tools in comparisons between inbreeding coefficients of parents and their progeny, and have been used very effectively in the study of mixed mating systems (Kohn and Barrett, 1994; Chang and Rausher, 1998; Fishman, 2000; Ritland, 2002; Ashley, 2010; Wang, El-Kassaby, and Ritland, 2012). Recently, caution has been advocated against the use of single populations for such studies owing to widespread variability in outcrossing rates between populations within species (Whitehead et al., 2018).

SELF-INCOMPATIBILITY

Circa 72% of all angiosperms are thought to possess hermaphroditic mating systems (de Jong and Klinkhamer, 2006), yet as Darwin illustrated in his thesis "*the effects of cross and self-fertilization in the vegetable kingdom*", this mating organisation presents a fundamental issue to plants as "*nature abhors perpetual self-fertilization*" (Darwin, 1876). Darwin was alluding to the reproductive costs of inbreeding depression. Inbreeding depression manifests through self-fertilization, where the progeny of cosexual species suffer significant reduction in fitness owing to the expression of recessive deleterious alleles in homozygous offspring (Charlesworth and Charlesworth, 1987). Because inbreeding depression has the potential to impose high reproductive and fitness costs on plants and their progeny respectively, it presents a significant selective force, and one which can strongly influence the evolution of mating systems (Darwin, 1876; Lloyd, 1979; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Devaux, Lande, and Porcher, 2014). In order to avoid the pitfalls of inbreeding, many angiosperms deploy a genetically controlled process of SI (self-incompatibility), which manifests through the active genetic recognition and rejection of male gametophytes (self-pollen or associated pollen-tubes) with *S*-alleles analogous to that of the female sporophyte (de Nettancourt, 1977, 1997, 2001).

Mating systems are strongly linked to a plant's life history, and short-lived annuals subject to poor habitats display a far greater incidence of selfing than perennial species (Munoz, Violle, and Cheptou, 2016; Barrett and Harder, 2017). Outcrossing offers favourable fitness gains, increasing heterozygosity, assisting in the prevention of deleterious recessive allele expression and increasing heterosis (Charlesworth and Willis, 2009; Barrett and Harder, 2017). Approximately 50% of angiosperms are believed to utilise mechanisms of SI in order to preclude self-fertilization (Darlington and Mather, 1949; Brewbaker, 1959; de Nettancourt, 1977). The ostensibly ubiquitous prevalence of SI in the flowering plants has led to suggestions that it arose frequently after angiosperm diversification, resulting in convergent evolution (Bateman, 1952) or alternatively evolved at an earlier stage, before angiosperms diverged (Whitehouse, 1951). Self-incompatibility has evolved in numerous families, many times in angiosperm history (de Nettancourt, 1977; Gibbs, 2014) and is a mating strategy which can be viewed as the evolutionary mitigation of inbreeding depression (Charlesworth and Charlesworth, 1987). Like pollinator-driven diversification, SI has been viewed as a mechanism central to the evolutionary diversification of the angiosperms due to its efficacy in the reinforcement of outcrossing (Whitehouse, 1951; de Nettancourt, 1977; Gibbs, 2014). In accordance with genetic, morphological and physiological variance, SI is conventionally categorised as either gametophytic or sporophytic or as homomorphic or heteromorphic (Heslop-Harrison, 1983; de Nettancourt, 1997; Barrett, 1998). Whilst different types of SI exist in plants, the majority of these mechanisms have in the past been thought to be prezygotic rather than postzygotic (de Nettancourt, 1977; Seavey and Bawa, 1986). Prezygotic SI mechanisms include, homomorphic gametophytic SI (GSI), homomorphic sporophytic SI (SSI) and heteromorphic SI (de Nettancourt, 1977; Seavey and Bawa, 1986). Homomorphic SSI is usually associated with tricellular self-pollen recognition and rejection on dry stigmas (de Nettancourt, 1977). Conversely, gametophytic SI and heteromorphic SI are usually associated with recognition and rejection of both bicellular and tricellular self-pollen on wet or dry stigmas, occurring variously in the stigma, style and ovary of the pistil (Sage, Pontieri, and Christopher, 2000).

Determining the location and temporal aspects of self-sterility in plants has important implications for understanding the overall functional significance of SI (Vaughton, Ramsey, and Johnson, 2010; Ford and Wilkinson, 2012), revealing the basis of SI (Sage et al., 1999) and for overriding SI in breeding applications (Hinata, Isogai, and Isuzugawa, 1994). Pinpointing the precise mechanism of post-pollination selection is often complicated and may be somewhat problematic, as selection affects the rate of selfing to such an extent that SI may vary between closely related taxa or even within populations of the same species (Jain, 1976; Whitehead et al., 2018). It has nonetheless been viewed essential to clarify the difference between the mechanisms of post-zygotic SI and those of the similar effects of inbreeding depression (Seavey and Bawa, 1986; Sage et al., 1999). This interpretation emanates from the widely held past view that any abortion of zygotes following self-pollination is more likely to be the work of early-acting inbreeding depression rather than that of post-zygotic SI (Seavey and Bawa, 1986; Sage et al., 1999; de Nettancourt, 2001). Although it may be difficult to distinguish the difference between the two phenomena, it can be attempted through pollen chase studies (Krebs and Hancock, 1990; Hokanson, 2000), the examination of cross-and self-pollen pollen-tube growth, the investigation of initial embryo or early seed development as well as the timing of zygote abortion (Seavey and Bawa, 1986; Sage et al., 1999; Sage, Price, and Waser, 2006; Johnson, Butler, and Robertson, 2019). Early-acting inbreeding depression typically results in the abortion of embryos at various developmental milestones whilst postzygotic SI manifests as uniform zygote termination prior to any further development (Charlesworth, 1985; Seavey and Bawa, 1986; Sage et al., 1999; de Nettancourt, 2001).

Investigation into angiosperm incompatibility is beginning to shed light on the previously marginalised phenomena known variously as, late-acting self-incompatibility (LSI; Seavey and Bawa, 1986), late-acting ovarian SI (OSI) or simply “ovarian sterility” (OS; Sage, Bertin, and Williams, 1994), where self-rejection occurs not in the stigma or style as in other forms of SI but rather in the ovary (Sears, 1937; Knight and Rogers, 1955; Cope, 1962; Dulberger, 1964; de Nettancourt, 1977; Kenrick, Kaul, and Williams, 1986; Seavey and Bawa, 1986; Sage, Bertin, and Williams, 1994; Gibbs, 2014). With LSI, pollen-tubes from both cross- and self-pollen travel to the ovary unhindered and yet, subsequent rejection of self-pollen tubes results in the abortion of fruit (Seavey and Bawa, 1986; Sage, Bertin, and Williams, 1994). Since rejection may supervene at any developmental stage from the integument of the ovule to the commencement of embryogenesis, termination of self-gametes could result in ovary abscission (Ford and Wilkinson, 2012). Late-acting self-incompatibility is characterised, quite distinctly, by the uniform failure of zygotes prior to embryogenesis and yet it can be notoriously difficult to distinguish it from early-acting inbreeding depression which manifests as the abortion of embryo's (homozygous for deleterious recessive alleles) at essentially any stage of development (Charlesworth, 1985; Seavey and Bawa, 1986; Charlesworth and Charlesworth, 1987; Hao et al., 2012).

Research from the past three decades indicates that LSI may operate both post-zygotically (Sage and Williams, 1991; Gibbs and Bianchi, 1993; Gibbs, Oliveira, and Bianchi, 1999; Bittencourt, Gibbs, and Semir, 2003; Sage and Sampson, 2003; Bittencourt and Semir, 2005) as well as pre-zygotically (Kenrick, Kaul, and Williams, 1986; Beardsell, Knox, and Williams, 1993; Sage et al., 1999; Sage, Price, and Waser, 2006; Chen et al., 2012; Hao et al., 2012), however the genetics of LSI are not yet well understood and few studies involve the underlying mechanisms of genetic control (Cope, 1962; Lipow and Wyatt, 2000; LaDoux and Friar, 2006). Numerous studies have identified species with LSI based virtually exclusively on comparisons between cross- and self-pollen tube development in the ovule (Gibbs and Bianchi, 1999; Gribel and Gibbs, 2002; Kawagoe and Suzuki, 2005; Vaughton, Ramsey, and Johnson, 2010), and increasingly it has been viewed as fundamental to identify the exact location and timing of self-rejection in order to fully comprehend the underlying genetic mechanisms of control (Sage et al., 1999; Ford and Wilkinson, 2012; Gibbs, 2014). Oftentimes in the past, the dearth of research into LSI's genetic basis of operation in itself has led to the dismissal of putative cases of LSI in favour of early-acting inbreeding-depression (Vaughton, Ramsey, and Johnson, 2010). In addition, confounding matters further, research has shown that LSI and early-acting inbreeding depression may even work together in unison and may well significantly influence plant mating system evolution (Goodwillie, Kalisz, and Eckert, 2005; Valtuena et al., 2010; Hao et al., 2012). With no general base of genetic studies from which to work, LSI remains somewhat contentious as much conjecture surrounds the actual mechanism involved in self-rejection (Sage, Bertin, and Williams, 1994; Sage et al., 1999). Because rejection occurs in the ovary it has been hypothesised that the control is of a gametophytic origin (Sage, Bertin, and Williams, 1994), however sporophytic SI cannot be ruled out despite the intricate nature of the long-distance extracellular signalling cascades which could be involved in the process (Sage et al., 1999).

There is great variation in the location as well as the timing of self-rejection in LSI systems (Seavey and Bawa, 1986). For example, research into the SI system of *Narcissus triandrus* L. (Amaryllidaceae) and *Ipomopsis aggregata* (Pursh) V.E. Grant (Polemoniaceae), indicate that ovule degeneration in self-pollinated flowers is prevalent even prior to ovule infiltration by pollen tubes, thus indicating the existence of a system of long-distance signalling (Sage et al., 1999; Sage, Price, and Waser, 2006). This form of LSI appears however to be an atypical example of prezygotic degeneration of female gametes (Sage et al., 1999; Sage, Price, and Waser, 2006). In *Thryptomene calycina* (Lindl.) Stapf (Myrtaceae), prezygotic LSI in manifests in the arrest of gametes in the micropyle or the placenta itself (Beardsell, Knox, and Williams, 1993). In *Theobroma cacao* L. (Malvaceae) recent findings obtained using live-cell confocal microscopy (Ford and Wilkinson, 2012) support earlier notions of an idiosyncratic form of LSI where gametes are prevented from uniting in the embryo sac (Cope, 1962), a LSI system shared with *Castanea mollissima* Blume (Fagaceae; McKay, 1942). More regularly, rejection of self-pollen tubes manifests as the failure of ovules to progress or the maturation of ovules up until the point that the pistils abscise (Dulberger, 1964; Sage and Williams, 1991; Gibbs and Bianchi, 1993; Gibbs and Bianchi, 1999; Bittencourt, Gibbs, and Semir, 2003; Sage and Sampson, 2003; Vaughton, Ramsey, and Johnson, 2010). Irrespective of the inherent physiological and molecular systems involved in LSI, there is an emerging view which identifies maternal prezygotic self-recognition as central to all systems of SI (Sage et al., 1999; Sage, Price, and Waser, 2006).

Research into LSI is beginning to lend recognition and legitimacy to this mating strategy as a unique form of SI in its own right (Seavey and Bawa, 1986; Sage et al., 1999; Sage, Price, and Waser, 2006; Vaughton, Ramsey, and Johnson, 2010; Chen et al., 2012; Ford and Wilkinson, 2012; Gibbs, 2014). There exists a very few pioneering studies which are beginning to shed light on the underlying mechanisms of genetic control (Cope, 1962; Lipow and Wyatt, 2000; LaDoux and Friar, 2006). In the Apocynaceae, work on *Asclepias exaltata* L. has revealed that LSI is controlled by multiple alleles on a single gene locus (Lipow and Wyatt, 2000). Conversely, research into SI systems of the Polemoniaceae indicate that the recognition of self-gametes in *Ipomopsis tenuifolia* (A.Gray) V.E. Grant requires the complete allelic matching of a minimum of three gene loci (LaDoux and Friar, 2006). In addition to genetic evidence, phylogenetic work on LSI provides substantiation for its existence as a distinct form of SI (Gibbs and Bianchi, 1999; Gibbs, 2014). Late-acting self-incompatibility appears to be clustered in particular plant families including notably, the Amaryllidaceae, Apocynaceae, Bignoniaceae, Malvaceae, Fabaceae and Xanthorrhoeaceae (Dulberger, 1964; Seavey and Bawa, 1986; Naaborgh and Willemse, 1991; Gibbs and Bianchi, 1999; Sage et al., 1999; Chase, 2004; Gibbs, 2014; Johnson, Butler and Robertson). Since a catholic phylogenetic distribution of LSI would be indicative of inbreeding depression rather than LSI, phylogenetic evidence offers additional credence to the legitimacy of LSI (Gibbs and Bianchi, 1999; Gibbs, 2014). It has therefore been proposed that studies of LSI should focus on the challenges of mapping phylogenetic distribution patterns, the genetic recognition of suitable mates and the identification of the site, mechanisms and timing of self-rejection (Barrett, 1998; Gibbs, 2014). It should also be emphasized that LSI must be carefully considered concurrently with the potential effects of inbreeding depression in order to determine the extent to which each phenomena contributes to overall plant fitness (Barrett, 1998; Gibbs, 2014).

Self-incompatibility bears its own distinctive reproductive costs and plants in possession of incompatibility systems may be more susceptible to pollen limitation than those of inbreeding taxa (Larson and Barrett, 2000; Knight et al., 2005). Plants with LSI may secure the fitness benefits associated with outbreeding, but they must also bear the reproductive costs associated with ovarian sterility which occur through the loss of available ovules, termed either “ovule usurpation” (Waser and Price, 1991) or “ovule discounting” (Barrett, Lloyd, and Arroyo, 1996). Following self-pollination, it is not just those species with LSI which face the risk of ovule discounting, but also those with strong early-acting inbreeding depression (Lloyd, 1992; Herlihy and Eckert, 2002). In addition, those species in possession of low ovule per flower ratios maybe particularly prone to ovule limitation (Vaughton, Ramsey, and Johnson, 2010). Numerous studies show that species with LSI display a severe reduction in seed set when stigmas are hand-pollinated with self-pollen either prior to or concurrently to that of cross-pollen (Cope, 1962; Dulberger, 1964; Waser and Price, 1991; Lloyd and Wells, 1992; Barrett, Lloyd, and Arroyo, 1996; Sage et al., 1999; Gribel and Gibbs, 2002; Vaughton and Ramsey, 2010; Vaughton, Ramsey, and Johnson, 2010; Johnson, Butler, and Robertson, 2019). Ovule limitation appears prevalently associated with LSI in that embryo production is often inadequate to exploit maternal resources due to ovule discounting (Sage et al., 1999; Harder and Aizen, 2010). When LSI is employed, female fecundity may be limited by loss of ovules to self-pollen tubes that otherwise would become seeds (Barrett, Lloyd, and Arroyo, 1996). Because ovule discounting has been shown to occur in numerous species exhibiting LSI (Cope, 1962; Dulberger, 1964; Waser and Price, 1991; Broyles and Wyatt, 1993; Sage et al., 1999), it is a mating strategy unique and potentially heavy reproductive costs (Layman et al., 2017) - a force magnified when copious amounts of self-pollen are received (Barrett, Lloyd, and Arroyo, 1996).

POLLEN LIMITATION AND ITS EVOLUTIONARY AND ECOLOGICAL IMPLICATIONS

Plant reproduction is intrinsically limited by adequate resources and limited extrinsically by suitable pollen and sufficient ovule numbers (Knight et al., 2005; Harder and Routley, 2006; Hove, Mazer, and Ivey, 2016). Pollination is fundamentally an unpredictable and stochastic process (Harder and Thomson, 1989), which is characteristically inefficient (Harder and Johnson, 2008). Since seed set is determined broadly by both male (pollen) and female contributions (available ovules and maternal energetics); plant reproduction is naturally limited by the weakest link in the chain (Harder and Routley, 2006). Resource limitation typically occurs when the actual number of embryos formed is lower than the potential number of seeds that can be produced, a phenomena occurring due to limited maternal energetic resources (Harder and Aizen, 2010; Hove, Mazer, and Ivey, 2016). Where pollinators become scarce, the occurrence of pollen limitation may become common, resulting in the reduction or failure of plants to set seed (Knight et al., 2005; Van Kleunen and Johnson, 2005; Ward and Johnson, 2005; Fishman and Willis, 2008). Inadequate pollen quantity (Ashman et al., 2004) and quality (Aizen and Harder, 2007), forms the basis of pollen limitation, whereby seed-set is reduced relative to the potential number of ovules available (Knight et al., 2005; Aizen and Harder, 2007). In other words, when pollen is of poor quality or insufficient quantity to fertilize all available ovules a plant is said to be pollen-limited (Ashman et al., 2004; Knight et al., 2005; Aizen and Harder, 2007). Providing that all available ovules are fertilized but insufficient embryos are produced to use up accessible maternal resources, then reproduction will be ovule-limited (Harder and Aizen, 2010).

In the past, the prevailing assumption has been that maternal reproductive success is seldom pollen-limited, yet in one survey of 258 species, 62% were shown to sustain reduced seed set as a result of pollen limitation (Burd, 1994). Since pollen limitation influences mating systems by negating desirable gene transmission, it may play a significant role in the adaptation and evolution of plant reproduction (Harder and Aizen, 2010). It has even been theorized that the minimum density thresholds for survival in plant populations can be determined by the degree of pollen limitation (Morgan, Wilson, and Knight, 2005). In another review investigating the fruit set of 482 data records, 63% were found to be subject to significant pollen limitation (Knight et al., 2005). Given the numerous variables involved in the uncertain pollination environment, it is not surprising that the probability of pollination is not uniform (Harder and Thomson, 1989). Breeding systems of plants governed by animal pollination vectors are therefore often subject to forces of evolutionary selection associated with variable pollination conditions (Harder and Barrett, 1996). The effect of pollen limitation on fitness can potentially be so great on plants that adaption can shift selection to favour those traits which improve the dispersal of pollen (male function) and favour the increased reception (female function) of good quality pollen in sufficient quantity (Ashman and Morgan, 2004; Harder and Aizen, 2010). Another outcome can be the evolution of autonomous self-pollination in self-compatible taxa, or even loss of self-incompatibility (Larson and Barrett, 2000; Eckert et al., 2010). Continued pollen limitation may impose such significant selective pressure on floral traits that the ensuing shift in traits results in speciation (Stebbins, 1970; Harder and Johnson, 2009). Evidence also suggests that the avoidance of inbreeding depression and mating system evolution, has contributed to the evolution of pollen limitation and that rather than simply being a constraint, pollen limitation represents a mechanism which has evolved in its own right (Devaux, Porcher, and Lande, 2019).

Variable floral visitation inherently causes temporal and spatial discrepancies in pollen limitation (Copland and Whelan, 1989). Continuous pollen limitation has been proposed as an evolutionary driving force shifting mating systems towards self-compatibility through plant reproductive assurance (Knight et al., 2005). The evolution of selfing in populations is however also opposed by the effects of pollen discounting and the associated effects of inbreeding depression (Porcher and Lande, 2005). Self-compatibility does however significantly benefit isolated individuals or small populations attracting few or infrequent pollinators (Van Kleunen and Johnson, 2005). There has also been some support indicating that pollen limitation may be lessened through increasing multiple mating opportunities (Larson and Barrett, 2000).

Curiously, despite the fact that numerous studies have shown a correlation between species in possession of LSI and the associated effects of ovule discounting (Cope, 1962; Dulberger, 1964; Waser and Price, 1991; Broyles and Wyatt, 1993; Sage et al., 1999), the extent to which the discounting of ovules in these taxa affects pollen limitation still needs to be addressed and for the most part remains unclear (Vaughton, Ramsey, and Johnson, 2010). One can nevertheless make an intuitive correlation between pollen limitation and ovule discounting. Pollen limitation is evaluated simply through the application of additional viable cross-pollen to stigmas of flowers which have been previously allowed to open naturally and are exposed only to the natural pollination environment (Aizen and Harder, 2007). If when testing for pollen limitation, the application of additional cross-pollen leads to an increase in seed set, then a species can be said to be subject to insufficient pollen quantities or quality in the wild

(Aizen and Harder, 2007). However the quality of pollen is no less of an issue to plants than is that of the pollen quantity and a large amount of self-pollen can diminish the overall quality of pollen received, which may reduce seed production due to ovule discounting (Ashman et al., 2004). Plants with LSI or early-acting inbreeding depression, being susceptible to ovule discounting (Waser and Price, 1991; Lloyd, 1992; Barrett, Lloyd, and Arroyo, 1996; Herlihy and Eckert, 2002), would in theory impose further "ovule precipitated-pollen limitation" occurring when incompatible pollen-tubes cause ovules to abort, negating any viable pollen preceding the self-rejection.

DISPERSAL AND RECALCITRANT SEEDS

Plant demography and evolution is immensely influenced by the abiotic and biotic mechanisms which affect seeds (Vander Wall et al., 2005). Seed removal and dispersal play an essential part in the long-term survival of species, influencing genetic structure, population dynamics and plant fitness (Howe and Smallwood, 1982; Wenny, 2001). Seed dispersal assists plants in avoiding density-dependant growth constraints, by reducing the effects of inbreeding, by aiding in seedling establishment of local microsites, and through facilitating movement into open habitats (Howe and Smallwood, 1982; Wenny, 2001; Howe and Miriti, 2004; Matthysen, 2012). Confirmation of the Janzen-Connell hypothesis – which suggests that density-dependant death of seeds or seedlings is negated by seed dispersal (Janzen, 1970; Connell, 1971) is beginning to emerge (Wills et al., 1997; Harms et al., 2000; Fricke, Tewksbury, and Rogers, 2014; Zhu et al., 2015), the studies of which are underpinned by a need for the integration of life history stages and species interactions when evaluating survival at both an individual as well as an ecological level (Zhu et al., 2015). Despite a wealth of historical research on seed dispersal ecology (van der Pijl, 1982; Fenner, 2000), a major limitation to such studies is that the seed dispersers of most plants and the effect they have on seedling establishment remain largely unknown.

The specific morphological characteristics of a species' seeds have classically been utilized by ecologists in the speculation of dispersal vectors, much like floral traits have been utilised in the formulation of pollination syndrome theories (Faegri and van der Pijl, 1979; van der Pijl, 1982). Animals however, exhibit a tremendous variety of foraging and feeding approaches, confounding definitive clarity in the formulation of putative 'seed-dispersal syndromes', with the consequence that models underpinning linkages between feeding, dispersal and the ultimate fate of seeds are in their minority (Howe, 1989; Lambert, 2002). The exploration of models linking plant evolutionary adaptation to animal dispersal vectors thus requires information on animal handling behaviour as well as the fate of seeds after primary dispersal (Howe, 1989; Lambert, 2002). Quantifying the extent to which seed distribution effects the dynamics of plant populations has also been challenging (Howe, 1989; Wang and Smith, 2002). Recruitment has all too often been examined in stages from a sequential perspective (Houle, 1995; Jordano and Herrera, 1995; Schupp and Fuentes, 1995; Wenny, 2001), often viewed diametrically - the botanical perspective focusing on stages of seedling development, with zoological approaches fixating on seed passage through the gut (Howe, 1993). Animals vary considerably in their effectiveness of seed dispersal (McKey, 1975; Howe and Estabrook, 1977; Wheelwright and Orians, 1982; Levey, 1987; Schupp, 1993). A measure of seed disperser effectiveness can be obtained by qualitative and quantitative mechanisms, where the former influenced by the nature of seed handling (including seed treatment in the mouth, gut and final deposition) and the latter influenced by the number visits a disperser makes to a plant and per-visit dispersal of seeds (Herrera and Jordano, 1981; Schupp, 1993). Previous views on tightknit evolution between dispersers and plants have given way that idea that co-evolution is not species-specific, but rather, represents varied adaptation to groups of dispersal agents (Wheelwright and Orians, 1982; Howe, 1984; Herrera, 1986). Although some plants have limited assemblages of dispersers (Janzen and Martin, 1982; Chapman, Chapman, and Wrangham, 1992; Tewksbury et al., 1999), no record exists for a plant reliant on one disperser species (Witmer and Cheke, 1991).

Recalcitrant, unorthodox or desiccation-sensitive seeds, are as the latter name implies, sensitive to desiccation both during growth and after development (Berjak and Pammenter, 2008). These seeds typically have a short lifespans ranging from a few days to a few months or a maximum of two years (Roberts, 1973; Pammenter and Berjak, 2000; Berjak and Pammenter, 2008). About 8% of seed plants produce seeds sensitive to desiccation (Wyse and Dickie, 2017), but the ecology and evolutionary significance of recalcitrant seeds has scarcely been understood (Pammenter and Berjak, 2000). Every major taxonomic group has species with desiccation-sensitive seeds and the trait has evolved a number of times in different lineages (Berjak and Pammenter, 2008; Costa et al., 2016; Wyse and Dickie, 2017; Subbiah et al., 2019). At lower taxonomic levels the trait is relatively conserved (Wyse and Dickie, 2017), with some families such as the Amaryllidaceae displaying a very high incidence of seed recalcitrance (Koopowitz, 1986; Meerow and Snijman, 1998). Plants with unorthodox seeds are more common in mesic habitats such as the tropics and subtropics (Berjak and Pammenter, 2008; Wyse and Dickie, 2017; Subbiah et al., 2019) compared to temperate regions, although descriptions of species with recalcitrance are even found in dryland habitats (Danthu et al., 2000; Pritchard et al., 2004). It is common for recalcitrant seeds to mature during the rainy season (Farnsworth, 2000) and a recent study found that approximately 18.5% of the plants in tropical and subtropical moist broadleaf forests possess desiccation-sensitive seeds (Wyse and Dickie, 2017). It has been proposed that the occurrence of numerous recalcitrant seeded species in wet and warm habitats illustrates resource saving though avoiding the costs associated with mechanisms of desiccation tolerance (Subbiah et al., 2019).

Recalcitrant seeds are commonly larger than orthodox seeds and this may offer a selective advantage by increasing the speed of germination, thereby reducing the risk of pathogen and predator attack, whilst expediting seedling establishment (Tweddle et al., 2003; Pritchard et al., 2004; Daws, Garwood, and Pritchard, 2005; Berjak and Pammenter, 2008). In moist tropical forests where growth rates are typically rapid and competition is fierce, any traits accelerating seedling establishment would be expected to be selected for. Increased seed size typically provides recalcitrant seeds with more water (Joët et al., 2016) and is also reflective of reserve storage, reserves which allow for not only faster germination, but also permit prolonged stages of seedling growth and establishment (Walters et al., 2013). Seedlings from species with large seeds generally have higher seedling survival rates compared to those of smaller seeds (Dalling and Hubbell, 2002; Moles and Westoby, 2004). One trade-off is that in general, larger seeds tend to have a lower vagility compared to smaller seeds, but because they are often allied with high competition as a consequence of habitat saturation (Werner, 1976), the trait is likely to be under significant selection pressure. Seed size has captivated the attention of ecologists (Salisbury, 1942; Leishman and Westoby, 1994; Rees, 1996; Westoby, Leishman, and Lord, 1996), with research historically concentrating on comparisons of functional groups, but studies are now being directed towards clarifying the diversity of seed sizes within functional groups (Grubb and Metcalfe, 1996; Rees and Westoby, 1997; Geritz, van der Meijden, and Metz, 1999; Leishman and Murray, 2001; Coomes and Grubb, 2003). Because the size of a seed and its ability for dispersal tend to be strongly linked (Harper, Lovell, and Moore, 1970), weedy plants and those growing in open habitats generally possess smaller seeds compared to those plants which reside in stable habitats and are long lived such as forest species (Levin and Kerster, 1974). The large seeds of recalcitrant-seeded species are unlikely to survive ingestion owing to the lack of a hard seed coat, but the link between dispersal and seed physiology has scarcely been examined.

Recalcitrant seeds are encoded to germinate during or subsequent to shedding (Berjak and Pammenter, 2008). Lacking dormancy and the ability to await favourable germination conditions, recalcitrant seeds are adapted to habitats which are favourable to instantaneous germination such as those which are permanently waterlogged, or with predictable and stable rainy seasons, or ephemeral flood plains (Marques et al., 2018). It has been suggested that upwards of 45% of species in evergreen tropical rain forests have recalcitrant seeds (Tweddle et al., 2003; Hamilton et al., 2013). Animals have been estimated to disperse upwards of 95% of tropical seeds, playing a vital role in ecosystem function (Terborgh et al., 2002), and yet the mechanisms of biotic seed dispersal in recalcitrant seeded species remains poorly understood (Meerow and Snijman, 1998). A number of recalcitrant seeds have fruits that are suggestive of animal dispersal, but very little information is available about animal dispersal agents (van der Pijl, 1982; Meerow and Snijman, 1998). Perhaps one of the few and most striking reports of animal consumption of fruit pulp in a recalcitrant species comes from reports of Jaguar consumption of *Persea gratissima* C.F.Gaertn. (van der Pijl, 1982). The removal of fruit pulp by frugivores, typically has effects on seed germination that are similar to those of gut passage (Barnea, Yomtov, and Friedman, 1991; Meyer and Witmer, 1998; Jordaan, Johnson, and Downs, 2011; Peña-Egaña, Loayza, and Squeo, 2018). De-pulping of fruit may assist germination (Evenari, 1949; Jordaan, Johnson, and Downs, 2011, 2012; Peña-Egaña, Loayza, and Squeo, 2018) and decrease attack from bacterial and fungal pathogens (Witmer and Cheke, 1991; Moore, 2001), conferring an advantage to plant fitness. Although gene flow through seed dispersal is limited in the tropics because long-distance dispersal mechanisms are not commonly developed (Levin and Kerster, 1974), and because seed caches are established close to their origin in animals with limited home ranges (Connell and Orias, 1964; van der Pijl, 1969), there are nevertheless clear advantages to animal dispersal.

Seed swallowing through fruit consumption or seed regurgitation in animal dispersal agents is generally considered to convey more effective seed dispersal compared to those dispersers which simply consume fruits (Jordano and Schupp, 2000), but this again applies to orthodox seeds that are able to survive ingestion. For the numerous recalcitrant seeded species that possess brightly coloured, metabolically expensive fruit rewards, suggestive of animal dispersal, restrictions to seed consumption in these species are likely to be provided for by unpalatable or toxic seeds. A great deal of species with recalcitrant seeds are vulnerable to habitat loss and over utilization (Berjak, 2005) and research into recalcitrance may provide conservation guidelines for management (Berjak and Pammenter, 2008; Wyse and Dickie, 2017), notwithstanding the provision for an ecological framework incorporating dispersal systems into recalcitrant seed reproductive biology.

STUDY SYSTEM AND RAISON D'ÊTRE

Southern Africa is home to one of the richest collections of temperate flora on earth, with some 368 families, containing roughly 24 000 taxa (Germishuizen and Meyer, 2003). The region comprises a meagre two and a half percent of the earth's surface and yet is home to more than 10% of the world's vascular plants (Germishuizen and Meyer, 2003). South Africa is a country of profuse floral wealth, and contains a number of biodiversity hotspots and World Heritage sites, with eight major biomes (Rutheford, 1997) and 14 centres of plant diversity (Davis, Heywood, and Hamilton, 1994; Van Wyk and Smith, 2001). Some have suggested that the dramatic variation in species richness found in Sub-Saharan Africa is a product of modern rainfall patterns, whilst the regions high levels of species endemism can been correlated with palaeoclimatic instability (Linder, 2001). Conversely, it has been suggested that regardless of precipitation, the advent of relatively continuous stable climactic conditions would favour the formation of species and centres of endemism (Fjeldså and Lovett, 1997). Some authors have suggested that speciation in the Greater Cape Floristic Region was a result of ecological alterations (Van der Niet and Johnson, 2009). Indeed, much evidence describing global diversification and speciation supports the idea that ecological transformations are at the heart of evolutionary process (Schluter, 2000; Rieseberg et al., 2002; Van der Niet and Johnson, 2009).

The enormous species diversity and extraordinarily high levels of endemism make South Africa an ideal place for delving into the workings of angiosperm adaptation and speciation. Southern Africa contains a large number of highly specialised pollination systems (Johnson and Steiner, 2000; Johnson and Steiner, 2003; Goldblatt and Manning, 2006; Johnson and Wester, 2017) and yet many of these pollination systems are poorly understood and inadequately described. This dissertation examines the biology of pollination and seed dispersal of the southern African endemic forest genus *Clivia* Lindley (Amaryllidaceae; see Figure 1), a handful of evergreen perennials within the African tribe Haemantheae (Meerow et al., 1999; Rourke, 2002). Phylogenetic analyses indicate that there was a major floral transition from pendulous to upright-trumpet shaped flowers in *Clivia* (Conrad, 2008; Conrad and Snijman, 2011) and the genus presents an ideal model for investigating pollinator-driven diversification and reproductive biology in a long-lived forest understory species subject to habitat fragmentation.

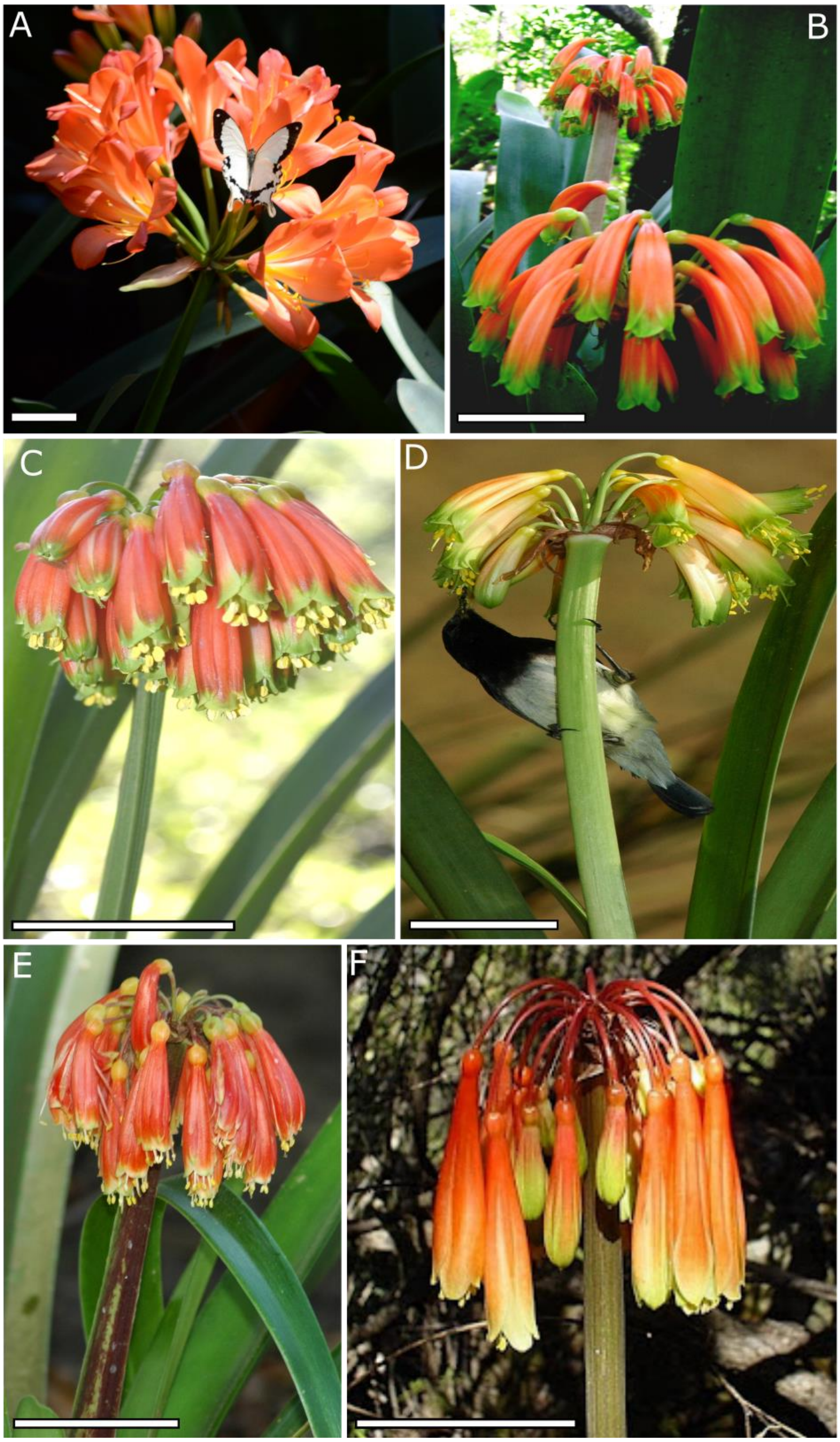

Figure 1. Flowers of the six described *Clivia* species. (A) *Clivia miniata* in the UNR, with alighting male *Papilio dardanus cena*. Photo: author. (B) *Clivia gardenii* "robust form" in swamp forest of the PC, showing two inflorescences on a single individual. Photo: author. (C) *Clivia caulescens*. Photo: author. (D) *Cinnyris talatala* probing a *C. gardenii* flower in the Pietermaritzburg area. Photo: S. D. Johnson. (E) *Clivia nobilis*. Photo: S. D. Johnson. (F) *Clivia mirabilis* in Oorlogskloof Nature Reserve. Photo: H. Grebe. Scale bars represent 40 mm.

THE AMARYLLIDACEAE

Although not a particularly large family, comprising approximately 75 genera and roughly 1600 species (Xu and Chang, 2017), the Amaryllidaceae are widely distributed throughout the world (Conrad et al., 2006). Concentrated centres of endemism are found in Andean South America, the Mediterranean and a large concentration of taxa in South Africa (Dahlgren, Clifford, and Yeo, 1985; Meerow and Snijman, 1998; Conrad et al., 2006). The Amaryllideae are spread throughout Africa (with the exception of the genus *Crinum* L., which occurs not just in Africa but also America, Australia and southern Asia) as does the Haemantheae (Dahlgren, Clifford, and Yeo, 1985).

THE GENUS *CLIVIA*

Clivia miniata is the only member of the genus with upright trumpet-shaped flowers whilst all other species possess pendulous tubular flowers. Until fairly recently it was thought that *Clivia* comprised of only four species, *C. miniata* (Lindl.) Bosse, *C. nobilis* Lindl. *C. gardenii* Hook. and *C. caulescens* R.A. Dyer (Rourke, 2002). In 2002, a fifth species *C. mirabilis* Rourke was described in the Northern Cape in a semi-arid habitat with a Mediterranean-like climate (Rourke, 2002). Two years later a sixth species *C. robusta* B.G. Murray, Ran, De Lange, Hammett, Truter *et* Swanevelder was proposed based on studies of plants from the Pondoland Centre of Endemism (PC) on the east coast of South Africa (Murray et al., 2004). The PC covers an area of 1880km^2 and is home to over 120 endemic or near endemic species (Van Wyk and Smith, 2001). Together with the Maputaland Centre, the Pondoland Centre represents two significant centres of floral endemism in Africa (Van Wyk, 1994). *Clivia robusta* was designated as a new species and not simply a "robust" form of *C. gardenii* on the basis of distinguishing traits including, dissimilar growth habit, habitat, vegetative and floral morphology and dissimilar karyology (Murray et al., 2004). However according to research, where *C. gardenii* and *C. robusta* occur sympatrically they possess considerable haplotype sharing (Conrad, 2008; Conrad and Snijman, 2011). The authenticity of *C. robusta* as a new species is also questioned by sequence data which do not provide evidence that *C. robusta* represents a distinct lineage (Conrad, 2008; Spies, Grobler, and Spies, 2011; Spies and Spies, 2018). Furthermore, there have not been any thorough studies of the extent of morphological discontinuities between the taxa in natural populations. Therefore, for the purposes of this dissertation I treat *C. robusta* as merely a "robust" form of *C. gardenii.* Although taxonomists are divided over whether or not to accept *C. robusta* as a valid taxon (Rourke, 2012), the description of two new species in little more than a decade, has led to a renewed scientific and horticultural interest in the genus.

The currently described natural distribution of *Clivia* ranges from Swaziland into South Africa through Mpumalanga, into the Northern Province, southwards into KwaZulu-Natal and into the coastal region of the Eastern Cape (Meerow and Snijman, 1998; Winter, 2000; Swanevelder and Fisher, 2009). The discovery of the rare and endangered *C. mirabilis* (Conrad, Reeves, and Rourke, 2003; Conrad et al., 2006; Conrad, 2008), extends the known range by some 800 km westwards into the Northern Cape

(Rourke, 2002) and Western Cape Provinces (Swanevelder and Fisher, 2009). *Clivia* species are highly adaptable and may be found in a diverse array of locations and microhabitats ranging from Mediterranean-type conditions to coastal, inland, as well as swamp-forests, riverbanks, cliffs and epiphytic environments on rocks and trees (Swanevelder, 2003; Murray et al., 2004). With the exception of *Clivia mirabilis*, the genus is found in coastal and Afromontane forest habitats and accordingly favours cool, shady and moist habitats (Duncan, 1999; Winter, 2000; Murray et al., 2004; Swanevelder and Fisher, 2009).

DISTRIBUTION AND FLOWERING PHENOLOGY OF *CLIVIA* SPECIES

Four of the *Clivia* species (five if *C. robusta* is recognized) are found in regions of summer rainfall, the exception being *C. mirabilis*, which is situated in the winter rainfall region of South Africa (Duncan, 1999; Winter, 2000; Koopowitz, 2002; Rourke, 2002; Duncan, 2004; Conrad, 2008; Swanevelder and Fisher, 2009; Conrad and Snijman, 2011). *Clivia nobilis* was the first species discovered and was described in 1828 (Koopowitz, 2002). *Clivia nobilis* is endemic to the south-east coast (Eastern Cape Province) of South Africa (between, 31ºS, 26ºE and 34ºS, 30ºE), and can be found predominantly growing on sandstone and shale outcrops as well as secondary coastal dunes (van der Merwe, Robbertse, and de Kock, 2005). *Clivia nobilis* flowers from spring to summer (August to January) and has been found growing as far inland as Grahamstown (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005). *Clivia gardenii* was described in 1856 and originates from the eastern regions (between 28ºS, 30ºE and 31ºS, 32.5ºE) of South Africa (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005). *Clivia gardenii* has been found as far south as Port St Johns and as far north as the northern boundary between KwaZulu-Natal and Swaziland (Winter, 2000). *Clivia gardenii* flowers from late autumn to mid-winter (May to July) and occurs on humic scree associated with sandstone outcrops (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005). *Clivia miniata* is the most widely distributed of the six recognised species (Winter, 2000; van der Merwe, Robbertse, and de Kock, 2005) and was described as we know it in 1864 (Koopowitz, 2002). Endemic to the eastern regions of South Africa (between 25.5ºS, 30ºE and 31ºS, 32ºE), *C. miniata* grows associated with substrates composed of sandstone and doleritic humic scree in a wide variety of conditions, from coastal to subtropical regions (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005). *Clivia miniata* is a spring (Austral) flowering species, with blooming ranging from August to early November (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005). *Clivia caulescens* was described in 1943 and is endemic to Mpumalanga and Limpopo Province (South Africa) as well as parts of Swaziland (between 23ºS, 30ºE and 25.5ºS, 30ºE) (Duncan, 1999; Winter, 2000; Swanevelder and Fisher, 2009). *Clivia caulescens* is fairly common and can be found in a number of different habitats ranging from humus rich loam associated with sandstone, to an epiphytic existence in trees and sandstone outcrops (Winter, 2000; van der Merwe, Robbertse, and de Kock, 2005). *Clivia caulescens* flowers in the Austral spring or from September to November (van der Merwe, Robbertse, and de Kock, 2005). *Clivia mirabilis*, first discovered in 2002, is the only species in the genus found associated with a semi-arid Mediterranean-like climate (Rourke, 2002; Murray et al., 2004). *Clivia mirabilis* is known only from a few locations, including the Oorlogskloof Nature Reserve in the Northern Cape Province and more recently, from isolated populations as far south as Clanwilliam (situated in the Cape Floristic Region) in the Western Cape Province of South Africa (Swanevelder and Fisher, 2009). *Clivia mirabilis* flowers in late spring or from October to mid-November (van der Merwe, Robbertse, and de Kock, 2005). *Clivia robusta* was described in 2004 and is also known as the swamp *Clivia* (or '*robust gardenii'*) as it is thought to be the only species in the genus able to tolerate wet swamp-like conditions (Murray et al., 2004). *Clivia robusta* is restricted to forests in the PC and favours the acidic, highly leached, sandy soils associated with this area (Van Wyk, 1994; Murray et al., 2004). *Clivia robusta* flowers from late March to early August or during autumn to winter (Murray et al., 2004).

PHYLOGENY OF *CLIVIA*

Clivia taxonomy has been the subject of a great deal of research in the past two decades (for a review see Spies, Grobler, and Spies, 2011) and was reinvigorated by the description of *C. mirabilis* (Rourke, 2002) and *C. robusta* (Murray et al., 2004). *Clivia* has been resolved as being a monophyletic clade sister to that of *Cryptostephanus* Welw. ex Baker (Meerow et al., 1999). The monophyletic group containing *Clivia* and *Cryptostephanus* was found to be sister to a clade containing *Haemanthus* L., *Scadoxus* Raf. and *Gethyllis* L. (Meerow et al., 1999). *Cryptostephanus* is a rhizomatous genus originating from east and central Africa, but is not present in Southern Africa (Conrad et al., 2006). *Cryptostephanus* represents a genus of baccate (berry-like)-fruited Haemantheae which, like *Clivia* is a forest understory taxon which does not form bulbs (Meerow et al., 1999).

Early work on the *Clivia* concentrated on identifying differences in chromosome numbers using karyotype analysis (Inariyama, 1937; Sato, 1938; Gouws, 1949). Research indicated chromosome numbers of the then described *Clivia* species (*C. nobilis*, *C. miniata*, *C. caulescens*, *C. gardenii*) to be 2n = 22 (Inariyama, 1937; Gouws, 1949), with the exception of *C. nobilis* which, was described as a tetraploid (Sato, 1938). Chromosome analysis and species characterization was undertaken more recently using Giemsa and fluorochrome banding, corroborating some of the earlier reports of diploid chromosome number (Ran, Murray, and Hammett, 1999). Although not all of the chromosome pairs were identified, the authors used banding patterns, measurements of length, and chromosome arm ratio's to characterize *Clivia* species, resulting in the differentiation of the genus into two groups; one containing *C. nobilis* and *C. caulescens* and the other containing *C. miniata* and *C. gardenii* (Ran, Murray, and Hammett, 1999).

The first phylogenetic analysis of *Clivia* was based on variation in DNA sequences, using ITS (internal transcribed spacer of 45S rDNA) and nuclear ribosomal 5S non-transcribed spacer sequences and reported a close relationship between *C. miniata* and *C. gardenii*, whilst "robust gardenii' was presented as a clade, sister to this group (Ran, Hammett, and Murray, 2001b). The authors suggested that *C. caulescens* was more closely related to this group than *C. nobilis* (Ran, Hammett, and Murray, 2001b). The discovery of *C. mirabilis* (Rourke, 2002), prompted the reassessment of phylogenetic relationships. Using four regions of non-coding plastid DNA, a subsequent study placed *C. mirabilis* as sister to the clade consisting of *C. nobilis, C. gardenii, C. miniata,* and *C. caulescens* (Conrad, Reeves, and Rourke, 2003). Unlike the phylogeny produced using 5S non-transcribed and ITS internal transcribed spacer regions, which placed "robust gardenii" as sister to the clade of *C. gardenii* and *C. miniata* (Ran, Hammett, and Murray, 2001b), the work using plastid sequences placed *C. gardenii* as a sister to a clade consisting of *C. miniata* and *C. caulescens* (Conrad, Reeves, and Rourke, 2003). Notably, this study suggested that the position of *C. miniata* was indicative of the ancestral state of pendulous flowers, and that the derived state of upright flowers suggest psychophilly to be a recent bifurcation from the ancestral condition of sunbird pollination (Conrad, Reeves, and Rourke, 2003). However, these early phylogenetic studies proved to be problematic as they omitted either *C. robusta* or *C. mirabilis* and as there was the additional possibility of species misidentification in some analyses.

More recent work incorporating all six described species, found that accessions of *C. mirabilis* and *C. nobilis* were monophyletic (Conrad and Snijman, 2011). These analyses suggest that the earliest divergence in the genus separates *C. mirabilis* from the remaining taxa, whilst *C. nobilis* was placed sister to a clade comprising *C. miniata, C. caulescens, C. gardenii,* and *C. robusta* (Conrad et al., 2006; Conrad and Snijman, 2011). The latter clade showed phylogenetic structure, but the species within it did not form clearly monophyletic lineages. The use of plastid sequence data to determine the relationships within the Haemantheae, identified *C. miniata* and *C. caulescens* to be sister taxa (Conrad et al., 2006), but another version of this analysis rather suggests that *C. miniata* is related to *C. gardenii* (Conrad and Snijman, 2011). It has been suggested that the position of *C. mirabilis* (Conrad, Reeves, and Rourke, 2003) indicates that an ancestral *Clivia* lineage once occupied the whole of southern Africa or has expanded from the Western Cape (present day distribution of *C. mirabilis*) in an easterly direction (Conrad et al., 2006). The present day distribution of *C. mirabilis* is thought to have been shaped by two possible developments. One scenario suggests that subsequent to the development of a Mediterranean-type climate, fire posed a major threat to forests in the south-western Cape, isolating the Northern Cape ancestors from the east coast of South Africa (Snijman, 2003). Another possible scenario suggests that *C. mirabilis* is a relictual of a bygone age, when much of central South Africa was covered in subtropical vegetation (Rourke, 2002). Given that the nearest relative of *C mirabilis, C. nobilis* lies over 800km to the east it is unlikely that long-distance dispersal of seed through birds was possible (Rourke, 2002). Molecular dating of the group indicates that *C. mirabilis* diverged from the summer rainfall species about the time of the Miocene (16 million years ago), when subtropical vegetation was destroyed as a result of increasing aridification (Conrad and Snijman, 2011).

Although very few reports detail the existence of interspecific hybrids in the wild, they are not unknown (Koopowitz, 2002; Rourke, 2003). Some researchers recognise only *C. x nimbicola,* an intermediate between *C. miniata* and *C. caulescens* (Swanevelder, Truter, and van Wyk, 2006) as the only natural hybrid (Conrad and Snijman, 2011; Spies, Grobler, and Spies, 2011). The sympatric distribution of many *Clivia* species means that hybridization is a possibility (Conrad and Snijman, 2011), partiucally in those species whose flowering overlaps. *Clivia nobilis* has a distribution overlapping with that of *C. gardenii*, spreading eastwards through the Eastern Cape (Conrad et al., 2006), but flowering times do not overlap (Swanevelder and Fisher, 2009). *Clivia miniata* on the other hand has a very wide-spread distribution, overlapping the ranges of *C. nobilis*, *C. gardenii* and *C. caulescens* (Winter, 2000). *Clivia miniata* grows sympatrically with a robust form of *C. gardenii* (*C. robusta*) in the Eastern Cape, near to the border of KwaZulu-Natal (Conrad and Snijman, 2011), but flowering time of the two species usually differs by several months. Haplotype sharing has been suggested between *C. miniata* and *C. robusta* as well as between *C. miniata* and *C. caulescens* and between *C. gardenii* and *"C. robusta"* (Conrad and Snijman, 2011). Haplotype sharing between *C. gardenii* and the "swamp *Clivia*", *C. robusta*, presents a particularly contentious issue, because some consider *C. robusta* to be simply a "robust" form of *C. gardenii* (Conrad and Snijman, 2011; Rourke, 2012).

Yet another, more recent phylogeny, constructed based on two DNA barcode sequences (*rbcLa* and *matK*) using Bayesian inference, suggests *C. caulescens*, *C. mirabilis* and *C. nobilis* are monophyletic, whilst *C. gardenii*, *C. robusta* and *C. miniata* are paraphyletic (Spies and Spies, 2018). This study concluded that *Clivia* is made up of 17 haplogroups, with *C. nobilis* and *C. mirabilis* residing in a single group (Spies and Spies, 2018). Although these results corroborate earlier findings that *C. gardenii*, *C. robusta* and *C. miniata* are closely related (Ran, Hammett, and Murray, 2001b), the authors have proposed a collective term of "*C. gardenii* complex" for the three described species (Spies and Spies, 2018). The authors call for a taxonomic revision, and additionally, suggest that barcode analysis indicates that *C. gardenii* specimens from Ngome represent a new species (Spies and Spies, 2018). Various hypothesis have been put forward to account for the phylogenetic incongruences and haplotype sharing in *Clivia*, ranging from ancestral polymorphisms and incomplete lineage sorting, to hybridization to (Conrad and Snijman, 2011). A major concern regarding the recent DNA barcode analysis (Spies and Spies, 2018) is that the study was undertaken using plant material from private collections. This is suboptimal practise for the construction of phylogenetic relationships of extant wild populations, and suggests that a comprehensive molecular study using material collected from wild populations would be required in order to fully disentangle the relationships in *Clivia*. A better resolved phylogeny of the genus is required, which could involve the use of additional molecular markers (Spies and Spies, 2018). Grouping *C. miniata*, *C. gardenii and C. robusta* into a single species complex (Spies and Spies, 2018) seems counterintuitive given that *C. miniata* and *C. gardenii* flower at different times and have dissimilar floral morphology and pollinators. Although the possibility of occasional hybridization between *C. miniata* and *C. gardenii* cannot be ruled out, flowering phenology, morphological and biochemical (scent) dissimilarity between these two taxa allude to separate species rather than that of a *"C. gardenii complex"*. The existence of *C. robusta* as discrete species remains highly debatable. Early reports identify the relationship between *C. robusta*, *C. gardenii* and *C. miniata* as unresolved (Spies, Grobler, and Spies, 2011), although most available phylogenies place *C. miniata* as a product of the most recent bifurcation in the genus (Ran, Hammett, and Murray, 2001b; Conrad, Reeves, and Rourke, 2003; Conrad et al., 2006; Conrad, 2008; Conrad and Snijman, 2011). To address some of these issues I embarked upon the use of SSR's (simple sequence repeats or microsatellites), but due to time constraints and difficulties encountered in genotyping, this work will be continued beyond the scope of this dissertation (see Appendix 4).

POLLINATION AND POLLINATOR SHIFTS IN *CLIVIA*

The description of two new *Clivia* species (Rourke, 2002; Murray et al., 2004), generated a renewed scientific interest in the genus, but studies focussed primarily on horticultural research or taxonomic delimitation (de Smedt, van Huylenbroeck, and Debergh, 1996; Meerow et al., 1999; Ran, Murray, and Hammett, 1999; Honiball, 2000; Meerow et al., 2000; Ran, 2000; Ran, Hammett, and Murray, 2001b, a; Ran, Murray, and Hammett, 2001; Conrad and Reeves, 2002; Conrad, Reeves, and Rourke, 2003; Swanevelder, 2003; Meerow and Clayton, 2004; Ran and Simpson, 2005; Swanevelder, Van Wyk, and Truter, 2005; Conrad et al., 2006; Swanevelder, Truter, and van Wyk, 2006; Conrad, 2008; Conrad and Snijman, 2011; Wang and Lei, 2012; Spies and Spies, 2018), with little attempts at ecological work (Middleton, 2017). A marked degree of anecdotal and speculative conjecture has surrounded the pollinators of *Clivia*. Given the immense popularity of the genus, it is surprising that pollinator observations have been exceptionally scarce (Koopowitz, 2002; Manning, 2005; Middleton, 2017). Descriptions of floral traits have been made for the purposes of phylogenies, comparative analysis and in order to draw inferences about pollinators based on floral syndromes (Duncan, 1999; Swanevelder, 2003; Manning, 2005; van der Merwe, Robbertse, and de Kock, 2005; Conrad, 2008; Swanevelder and Fisher, 2009; Conrad and Snijman, 2011). Most discussion has focused on floral syndromes (Koopowitz, 2002; Manning, 2005; Swanevelder and Fisher, 2009) and little attempt has been made to study *Clivia* pollination in any ecological framework (but see Middleton, 2017).

Clivia miniata flowers are consistent with a butterfly pollination syndrome whist those of the remainder of the genus are consistent with that of bird pollination (Table 1; Faegri and van der Pijl, 1979). Observations of pendulous flowered *Clivia* species suggest pollination by various species of sunbirds, whilst those of *C. miniata* suggest pollination by butterflies (Koopowitz, 2002; Manning, 2005; Swanevelder and Fisher, 2009). A handful of observations exist, including that of an olive sunbird (*Cyanomitra olivacea*) visiting *C. gardenii* in a coastal Kwazulu-Natal garden (Koopowitz, 2002), a black sunbird (*Chalcomitra amethystina*) visiting *C. caulescens* at God's Window in Mpumalanga Province, and a malachite sunbird (*Nectarinia famosa*) was reported visiting *C. mirabilis* at Oorlogskloof Nature Reserve in the Northern Cape (Manning, 2005). The latter observation was corroborated in October 2015 using video footage captured from three sites, depicting eight malachite sunbirds and one dusky sunbird (*Cinnyris fuscus*) feeding on *C. mirabilis* in Oorlogskloof gorge

(Middleton, 2017). Of noteworthy interest was that *C. fuscus* was observed piercing the corolla with its bill, effectively robbing flowers for nectar, and mutilated flowers were reported to be commonplace (Middleton, 2017). A citrus swallowtail (*Papilio demodocus demodocus*) was reported visiting *C. miniata* flowers at Morgan's Bay in the Eastern Cape Province (Manning, 2005). *Clivia miniata* flowers have been described as unscented for the most part, but some authors have suggested that certain varieties emit a "sweet floral fragrance" (Manning, 2005). Other authors have proposed moth pollination in *C. miniata*, suggesting that the trumpet-shaped flowers, central yellow "nectar guide" and the "azalea-like" fragrance are indicative of this mode of pollination (Swanevelder and Fisher, 2009). These authors nevertheless attest to the current lack of knowledge available on *Clivia* pollinators and speculate on the potential presence of some long extinct pollinator, possibly a butterfly (Swanevelder and Fisher, 2009). This thesis provides much needed clarification on pollination in the genus (Kiepiel and Johnson, 2014).

The existing anecdotal observations of swallowtail butterflies visiting *C. miniata* and sunbirds visiting the pendulous tubular flowered *Clivia* species together with floral trait data, lend credence to butterfly and bird pollination syndromes (Table 1) respectively (Duncan, 1999; Koopowitz, 2002; Manning, 2005; van der Merwe, Robbertse, and de Kock, 2005; Swanevelder and Fisher, 2009; Middleton, 2017). Based on floral syndrome inferences and an assortment of anecdotal observations, it has been suggested that this fundamental shift in floral morphology goes hand in hand with the shift from a predominantly bird to butterfly floral syndrome (Manning, 2005). Research from a number of plant lines show evidence of evolutionary shifts between bird and lepidopteran pollination (Perret et al., 2007; Whittall and Hodges, 2007; Tripp and Manos, 2008). A survey of the literature indicates that in comparison to shifts involving other Lepidoptera, shifts involving butterfly pollination are underrepresented and poorly documented (Van der Niet and Johnson, 2012). However, a number of studies undertaken on various plant families offer evidence for shifts between bird and lepidopteran pollination (Perret et al., 2007; Whittall and Hodges, 2007; Tripp and Manos, 2008; Gübitz et al., 2009). For example, the evolution of hawkmoth pollination from hummingbird pollination in *Aquilegia* L. (Whittall and Hodges, 2007), whilst in *Petunia* Juss. the opposite scenario occurred with at least one species evolving hummingbird pollination from the ancestral hawkmoth-pollinated condition (Gübitz et al., 2009). On the other hand, in some irid genera such as *Gladiolus* L. and *Tritoniopsis* L. Bolus, butterfly pollination for the most part, evolved from ancestral long-proboscid fly and long-tongued bee pollinated ancestors (Manning and Goldblatt, 2005; Valente et al., 2012).

Table 1. Comparison between butterfly and bird pollination syndromes, describing the floral traits of butterfly- and bird-pollinated flowers as well as the ethology and morphology of their corresponding pollinators (modified from Faegri and van der Pijl, 1966, 1979).

Psychophily (butterfly pollination)		Ornithophily (bird pollination)	
Butterflies	Butterfly blossoms	Birds	Bird blossoms
Diurnal	Diurnal anthesis	Diurnal	Diurnal anthesis
Olfactory sense not very strong	Odour weak, generally fresh agreeable	Scarcely any sense of smell	Absence of odours
Visual sense well-developed also for colours, some see red	Vividly coloured, including pure red	Visual with sensitivity for red	Vivid colours, often scarlet or with contrasting parrot colours
Alighting on blossoms or hovering supported by front legs. Probably not sensitive to deeply dissected contours	Blossom rim not much dissected. Blossom erect, radial, rim generally flat, but often narrow; anthers fixed	Too large to alight on the flower itself. Hovering (hummingbirds) or perching (other birds)	Lip or margin absent or curved back, flower tubate and/or hanging zygomorphy unnecessary
Long, thin proboscis	Nectar well hidden in tubes or spurs; tubes narrow	Long bill and tongue	Deep tube or spur, wider than in butterfly flowers. Distance between nectar-sexual sphere may be large
Less active flyer, metabolism not very high	Small amounts of dilute nectar	Large- and great consumers	Large amounts of dilute nectar
Some preference for guiding marks for inserting proboscis	Simple nectar guides or groove (mechanical tongue guide)	Intelligent at finding entrance	Nectar-guide absent or plain

MATING SYSTEMS OF THE AMARYLLIDACEAE

For the most part breeding systems of the Amaryllidaceae are unknown and poorly documented. This is particularly the case in the Southern Hemisphere where the majority of Amaryllids are found. Self-incompatibility has been found in a number of Amaryllidaceae (Sage et al., 1999; Arroyo et al., 2002; Perez-Barrales, Vargas, and Arroyo, 2006; Vaughton, Ramsey, and Johnson, 2010; Parolo et al., 2011; Navarro et al., 2012; Streher et al., 2018; Johnson, Butler, and Robertson, 2019). Early mating system studies suggest that the family possesses gametophytic SI (Pandey, 1960), whilst Amaryllids such as *Narcissus tazetta* L. (Dulberger, 1964), *N. triandrus* L. (Bateman, 1954; Sage et al., 1999), *N. cyclamineus* Redouté (Navarro et al., 2012), *N. papyraceus* Ker Gawl. (Simon, 2013; de Castro et al., 2015), *Cyrtanthus breviflorus* Harv. (Vaughton, Ramsey, and Johnson, 2010), *Cyrtanthus contractus* N.E.Br., *Cyrtanthus, ventricosus* Willd. and *Cyrtanthus mackenii* Hook.f (Johnson, Butler, and Robertson, 2019) have been reported to possess LSI. Recently, gametophytic SI has been reported in *Habranthus gracilifolius* Herb. (Streher et al., 2018). The genetic mechanisms involved in LSI in these species have yet to be clarified and further histological work is required to determine the timing of LSI in *C. breviflorus* (Vaughton, Ramsey, and Johnson, 2010). Research indicates that LSI acts pre-zygotically in *N. triandrus* (Sage et al., 1999) and post-zygotically in *N. tazetta* (Dulberger, 1964).

The breeding systems of *Clivia* have not been extensively researched and anecdotal accounts advocate partial SI (Koopowitz, 1986; Duncan, 1999; Koopowitz, 2002; Manning, 2005), with *C. mirabilis* producing up to three seeds per berry following self-pollination (Swanevelder and Fisher, 2009). Horticulturalists report a substantial reduction or total lack of seed set following self-pollination (Koopowitz, 1986; Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005), although self-pollination in certain lines has been described (Swanevelder and Fisher, 2009). Plants in natural populations have been reported to produce a lower seed set compared to cultivated plants (van der Merwe, Robbertse, and de Kock, 2005; Swanevelder and Fisher, 2009).

DISSERTATION OVERVIEW

This thesis uses *Clivia* as a model for exploring pollinator shifts, breeding systems, advertising signals and seed dispersal in an evolutionary framework. The breeding, mating and pollination systems of *C. miniata* and *C. gardenii* are investigated and reproductive biology is further explored through the investigation of floral trait adaptation and seed dispersal of *C. miniata*. This dissertation comprises of an introduction, the present chapter, which covers general literature pertinent to the studies undertaken herein and offers a contextual framework to the research presented (Chapter 1). Three discrete peer-reviewed publications (Chapter 2, 3 & 5) and an as yet unpublished manuscript (Chapter 4), offer new scientific contributions, whilst the concluding discussion relates the implications of this investigation and offers potential future research directives (Chapter 6). Following the concluding chapter is a brief discussion on novel molecular marker development in *Clivia* and the preliminary application of these SSR’s which is beyond the scope of this thesis (Appendix 4). Appendix 5 presents an unpublished manuscript which examines the relationship between floral curvature, orientation, and perch position in perching bird mutualisms, which is the result of collaborative work undertaken during the course of this research.

RESEARCH OBJECTIVES

Chapter 2 explores the concept that pollinator shifts are implicated as drivers of angiosperm diversification. I tested the hypothesis that a shift took place from bird to butterfly pollination in *Clivia*. The aims of this Chapter were: (1) to determine whether pendulous and upright trumpet-flowered *Clivia* species are as hypothesized, pollinated by sunbirds and butterflies respectively and, (2) to determine whether floral traits such as morphology, spectral reflectance, nectar and scent emissions were either modified or retained during the putative pollinator shift.

Chapter 3 investigates breeding and mating systems in *Clivia*, investigating SI and its potential functional consequences on plant fitness. I tested the hypothesis that *Clivia* is likely to possess LSI. The aims of this Chapter were: (1) to determine the level of self-fertility and potential for autonomous self-pollination in the genus; (2) to test whether self-pollen tubes enter the ovary and are able to penetrate the ovules; and (3) to determine whether self-pollination results in ovule discounting with an impact on fecundity.

Chapter 4 explores the functional significance of floral traits in *C. miniata* and adaptation to specialist butterfly pollination. The aims of this chapter were to identify the floral traits responsible for butterfly attraction to *C. miniata.* More specifically, I sought to determine the role of colour, nectar guides, size, shape, orientation and scent on butterfly attraction. I tested the hypotheses that: (1) butterflies will favour orange flowers with a central yellow target pattern over other colours and patterns, (2) that butterflies will be more attracted to, and more likely to settle on, flowers which face upwards, (3) that butterflies will show electrophysiological responses to scent compounds emitted by *C. miniata* and prefer scented over unscented flowers.

Chapter 5 describes dispersal and seed germination of *C. miniata*. I tested the hypothesis that seeds are dispersed by primates and that fruit de-pulping increases germination rates and results in fewer deaths than non-removal of fruit. The aims of this Chapter were: (1) to identify animals consuming *C. miniata* fruit, (2) to determine whether they separate seeds from fruit pulp prior to ingestion and (3) to study the effects of de-pulping on germination and the growth of seedlings.

Appendix 4 details the development and preliminary application of a novel array of SSR markers. The initial aims at the outset of this research were to use SSR markers to assess to assess phylogeographical and population genetic among populations of *C. gardenii* and *C. robusta* and to study outcrossing rates in *C. miniata* and *C. gardenii*, but due to complications and time constraints this work will be continued at a later stage and is beyond the scope of this thesis.

Appendix 5 describes the functional consequences of floral curvature and orientation relative to perch position for nectar feeding sunbirds. The hypothesis that perching nectarivorous birds such as sunbirds have decurved bills because they probe in an arc-like probing motion was tested. The aims of this research were to determine: (1) whether birds would prefer to land on "inflorescences" with flowers curved towards the perch, (2) whether birds would favour feeding from a perching position that enabled an arc-like probe into a curved flowers, (3) whether birds would probe flowers that curve towards the perch more than those that did not do so, (4) whether birds would consume more nectar from flowers that curved towards the perch and (5) whether birds would handle flowers that curved towards the perch more quickly than those that did not.

REFERENCES

ÅGREN, J., F. HELLSTRÖM, P. TORÄNG, AND J. EHRLÉN. 2013. Mutualists and antagonists drive among-population variation in selection and evolution of floral display in a perennial herb. *Proceedings of the National Academy of Sciences of the United States of America* 110: 18202–18207.

AIZEN, M. A., AND L. D. HARDER. 2007. Expanding the limits of the pollen-limitation concept: effect of pollen quantity and quality. *Ecology* 88: 271-281.

ALTHOFF, D. M., K. A. SEGRAVES, AND M. T. J. JOHNSON. 2014. Testing for coevolutionary diversification: linking pattern with process. *Trends in Ecology and Evolution* 29: 82-89.

ANDERSSON, S. 2003. Foraging responses in the butterflies *Inachis io*, *Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae) to floral scents. *Chemoecology* 13: 1-11.

ANDERSSON, S., L. A. NILSSON, I. GROTH, AND G. BERGSTROM. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Botanical Journal of the Linnean Society* 140: 129-153.

ARMBRUSTER, W. S. 1996. Evolution of floral morphology and function: an integrative approach to adaptation, constraint, and compromise in *Dalechampia* (Euphorbiaceae). *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal-pollinated plants, 241-272. Chapman and Hall, New York, USA.

ARROYO, J., S. C. H. BARRETT, R. HIDALGO, AND W. W. COLE. 2002. Evolutionary maintenance of stigma-height dimorphism in Narcissus papyraceus (Amaryllidaceae). *American Journal of Botany* 89: 1242-1249.

ASHLEY, M. V. 2010. Plant parentage, pollination, and dispersal: how DNA microsatellites have altered the landscape. *Critical Reviews in Plant Sciences* 29: 148-161.

ASHMAN, T.-L., AND M. STANTON. 1991. Seasonal variation in pollination dynamics of sexually dimorphic *Sidalcea oregana* spp. *spicata* (Malvaceae). *Ecology* 72: 993-1003.

ASHMAN, T. L., AND M. T. MORGAN. 2004. Explaining phenotypic selection on plant attractive characters: male function, gender balance or ecological context? *Proceedings of the Royal Society of London Series B-Biological Sciences* 271: 553-559.

ASHMAN, T. L., T. M. KNIGHT, J. A. STEETS, P. AMARASEKARE, M. BURD, D. R. CAMPBELL, M. R. DUDASH, et al. 2004. Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. *Ecology* 85: 2408-2421.

ASHWORTH, L., R. AGUILAR, S. MARTÉN-RODRÍGUEZ, M. LOPEZARAIZA-MIKEL, G. AVILA-SAKAR, V. ROSAS-GUERRERO, AND M. QUESADA. 2015. Pollination syndromes: a global pattern of convergent evolution driven by the most effective pollinator. *In* P. Pontarotti [ed.], Evolutionary Biology: Biodiversification from Genotype to Phenotype, 203-224. Springer International Publishing, Switzerland.

BAKER, H. G. 1963. Evolutionary mechanisms in pollination biology. *Science* 139: 877-883.

BAKER, H. G. 1979. Anthecology: old testament, new testament, apocrypha (banquet address, 8 February 1979). *New Zealand Journal of Botany* 17: 431-440.

BALDWIN, B. G. 1997. Adaptive radiation of the Hawaiian silversword alliance: congruence and conflict of phylogenetic evidence from molecular and non-molecular investigations. *In* T. Givnish AND K. J. Sytsma [eds.], Molecular evolution and adaptive radiation, 103-128. Cambridge University Press, Cambridge, UK.

BARNEA, A., Y. YOMTOV, AND J. FRIEDMAN. 1991. Does ingestion by birds affect seed-germination. *Functional Ecology* 5: 394-402.

BARRACLOUGH, T. G., A. P. VOGLER, AND P. H. HARVEY. 1998. Revealing the factors that promote speciation. *Philosophical Transactions of the Royal Society of London Series B* 353: 241-249.

BARRETT, S. C. H. 1995. Mating-system evolution in flowering plants: micro- and macroevolutionary approaches. *Acta Botanica Neerlandica* 44: 385-402.

BARRETT, S. C. H. 1998. The evolution of mating strategies in flowering plants. *Trends in Plant Science* 3: 335-341.

BARRETT, S. C. H. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274-284.

------. 2003. Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 358: 991-1004.

BARRETT, S. C. H. 2010. Understanding plant reproductive diveristy. *Philosophical Transactions of the Royal Society Series B* 365: 99-109.

BARRETT, S. C. H., AND B. C. HUSBAND. 1990. Variation in outcrossing rates in *Eichhornia paniculata*: the role of demographic and reproductive factors. *Plant Species Biology* 5: 41-55.

BARRETT, S. C. H., AND C. G. ECKERT. 1990. Variation and evolution of mating systems in seed plants. *In* S. Kawano [ed.], Biological Approaches and Evolutionary Trends in Plants, 229-254. Academic Press, London, UK.

BARRETT, S. C. H., AND L. D. HARDER. 1996. Ecology and evolution of plant mating. *Trends in Ecology & Evolution* 11: A73-A79.

------. 2017. The ecology of mating and its evoutionary consequences in seed plants. *Annual Review of Ecology and Systematics* 48: 135-157.

BARRETT, S. C. H., L. D. HARDER, AND A. C. WORLEY. 1996. The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences* 351: 1271-1280.

BARRETT, S. C. H., D. G. LLOYD, AND J. ARROYO. 1996. Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal pollinated plants 339-376. Chapman and Hall, New York, USA.

BARRETT, S. C. H., A. M. BAKER, AND L. K. JESSON. 2000. Mating strategies in monocotyledons. *In* K. L. Wilson AND D. A. Morrison [eds.], Monocots: systematics and evolution, 258-269. CSIRO, Melbourne, Australia.

BARRETT, S. C. H., W. W. COLE, AND C. M. HERRERA. 2004. Mating patterns and genetic diversity in the wild Daffodil *Narcissus longispathus* (Amaryllidaceae). *Heredity* 92: 459-465.

BATEMAN, A. J. 1952. Self-incompatibility systems in angiosperms. I. Theory. *Heredity* 6: 285-310.

------. 1954. The genetics of *Narcissus*. 1. Sterility. Daffodil and Tulip Year Book. *Royal Horticultural Society* 19: 23-29.

BEARDSELL, D. V., R. B. KNOX, AND E. G. WILLIAMS. 1993. Breeding system and reproductive success of *Thryptomene calycina* (Myrtaceae). *Australian Journal of Botany* 41: 333-353.

BEARDSLEY, P. M., A. YEN, AND R. G. OLMSTEAD. 2003. AFLP phylogeny of *Mimulus* section *Erythranthe* and the evolution of hummingbird pollination. *Evolution* 57: 1397-1410.

BELL, G. 1985. On the function of flowers. *Proceedings of the Royal Society of London, Series B, Biological Science* 224: 223-265.

BERGSTRÖM, G., H. E. M. DOBSON, AND I. GROTH. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). *Plant Systematics and Evolution* 195: 221-242.

BERJAK, P. 2005. Protector of the seeds: seminal reflections from southern Africa. *Science* 307: 47-49.

BERJAK, P., AND N. W. PAMMENTER. 2008. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. *Annals of Botany* 101: 213-228.

BERNARDELLO, G., G. J. ANDERSON, T. F. STUESSY, AND D. J. CRAWFORD. 2001. A survey of floral traits, breeding systems, floral visitors, and pollination systems of the angiosperms of the Juan Fernádez Islands (Chile). *Botanical Review* 67: 255-308.

BIESMEIJER, J. C., S. P. M. ROBERTS, M. REEMER, R. OHLEMULLER, M. EDWARDS, T. PEETERS, A. P. SCHAFFERS, et al. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* 313: 351-354.

BITTENCOURT, N. S., AND J. SEMIR. 2005. Late-acting self-incompatibility and other breeding systems in *Tabebuia* (Bignoniaceae). *International Journal of Plant Sciences* 166: 493-506.

BITTENCOURT, N. S., P. E. GIBBS, AND J. SEMIR. 2003. Histological study of post-pollination events in *Spathodea campanulata* Beauv. (Bignoniaceae), a species with late-acting self-incompatibility. *Annals of Botany* 91: 827-834.

BRADSHAW, H. D., AND D. W. SCHEMSKE. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426: 176-178.

BREITKOPF, H., R. E. ONSTEIN, D. CAFASSO, P. M. SCHLÜTER, AND S. COZZOLINO. 2015. Multiple shifts to different pollinators fuelled rapid diversification in sexually deceptive *Ophrys* orchids. *New Phytologist* 207: 377-389.

BREWBAKER, J. L. 1959. Biology of the angiosperm pollen grain. *Indian Journal of Genetics and Plant Breeding* 19: 121-133.

BRODY, A. K. 1997. Effects of pollinators, herbivores, and seed predators on flowering phenology. *Ecology* 78: 1624-1631.

BROYLES, S. B., AND R. WYATT. 1993. The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *American Journal of Botany* 80: 41-44.

BURD, M. 1994. Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review* 60: 83-139.

CAMPBELL, D. R., N. M. WASER, AND M. V. PRICE. 1996. Mechanisms of hummingbird-mediated selection for flower width in *Ipomopsis aggregata*. *Ecology* 77: 1463-1472.

CAMPBELL, D. R., N. M. WASER, AND E. J. MELÉNDEZ-ACKERMAN. 1997. Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *American Naturalist* 149: 295-315.

CAMPBELL, D. R., N. M. WASER, M. V. PRICE, E. A. LYNCH, AND R. J. MITCHELL. 1991. Components of phenotypic selection: pollen export and flower corolla width in *Ipomopsis aggregata*. *Evolution* 45: 1458-1467.

CAMPOS, E. O., H. D. BRADSHAW, AND T. L. DANIEL. 2015. Shape matters: corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. *Functional Ecology* 29: 462-468.

CARIVEAU, D., R. E. IRWIN, A. K. BRODY, L. S. GARCIA-MAYEYA, AND A. VON DER OHE. 2004. Direct and indirect effects of pollinators and seed predators to selection on plant and floral traits. *Oikos* 104: 15-26.

CARLSON, J. E., AND K. E. HOLSINGER. 2012. Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171: 905-919.

CARR, D. E., AND M. D. EUBANKS. 2014. Interactions between insect herbivores and plant mating systems. *Annual Review of Entomology* 59: 185-203.

CASPER, B. B., AND T. R. LA PINE. 1984. Changes in corolla color and other floral characteristics in *Cryptantha humilis* (Boraginaceae): cues to discourage pollinators? *Evolution* 38: 128-141.

CASTELLANOS, M. C., P. WILSON, AND J. D. THOMSON. 2004. 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* 17: 876-885.

CHANG, S. M., AND M. D. RAUSHER. 1998. Frequency-dependent pollen discounting contributes to maintenance of a mixed mating system in the common morning glory *Ipomoea purpurea*. *American Naturalist* 152: 671-683.

CHAPMAN, L. J., C. A. CHAPMAN, AND R. W. WRANGHAM. 1992. *Balanites wilsoniana* - elephant dependent dispersal. *Journal of Tropical Ecology* 8: 275-283.

CHARLESWORTH, D. 1985. Distribution of dioecy and self-incompatibility in angiosperms. *In* P. J. Greenwood, P. H. Harvey, AND M. Slatkin [eds.], Evolution: essays in honour of John Maynard Smith, 237-268. Cambridge University Press, Cambridge, UK.

CHARLESWORTH, D., AND B. CHARLESWORTH. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* 18: 237-268.

CHARLESWORTH, D., AND J. H. WILLIS. 2009. The genetics of inbreeding depression. *Nature Reviews Genetics* 10: 783-796.

CHASE, M. W. 2004. Monocot relationships: and overview. *American Journal of Botany* 91: 1645-1655.

CHEN, X., S. HAO, L. WANG, W. FANG, Y. WANG, AND X. LI. 2012. Late-acting self-incompatibility in tea plant (*Camellia sinensis*). *Biologia* 67: 347-351.

CHITTKA, L., AND R. MENZEL. 1992. The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *Journal of Comparative Physiology A (Sensory, Neural, and Behavioral Physiology)* 171: 171-181.

CHITTKA, L., AND N. E. RAINE. 2006. Recognition of flowers by pollinators. *Current Opinion in Plant Biology* 9: 428-435.

CHITTKA, L., J. D. THOMSON, AND N. M. WASER. 1999. Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86: 361-377.

COATES, D. J., J. F. SAMPSON, AND C. J. YATES. 2007. Plant mating systems and assessing population persistence in fragmented landscapes. *Australian Journal of Botany* 55: 239-249.

CONNELL, J. H. 1971. On the role of natural enemies in preventing competitive exclusion in some marine mammals and in rain forest trees. *In* P. J. Boer AND G. R. Gradwell [eds.], Dynamics of populations. PUDOC, Netherlands.

CONNELL, J. H., AND E. ORIAS. 1964. The ecological regulation of species diversity. *American Naturalist* 98: 399-413.

CONNER, J. K., AND S. RUSH. 1996. Effects of flower size and number on pollinator visitation to wild radish, *Raphanus raphanistrum*. *Oecologia* 105: 509-516.

CONRAD, F. 2008. Molecular systematics, biogeography and dating of the tribe Haemantheae (Amaryllidaceae) and the phylogeography of *Clivia*. PhD thesis, University of Cape Town, Cape Town, South Africa.

CONRAD, F., AND G. REEVES. 2002. Molecular systematics of the genus Clivia. *Clivia* 4: 20-23.

CONRAD, F., AND D. SNIJMAN. 2011. Systematics and Phylogeography of *Clivia*. *In* R. Fisher AND R. Dixon [eds.], Clivia Thirteen. *Clivia* Society, Pretoria, South Africa.

CONRAD, F., G. REEVES, AND J. P. ROURKE. 2003. Phylogenetic relationships of the recently discovered species - *Clivia mirabilis*. *South African Journal of Botany* 69: 204-206.

CONRAD, F., G. REEVES, D. A. SNIJMAN, AND T. A. J. HEDDERSON. 2006. Genetic relationships within the tribe Haemantheae (Amaryllidaceae) based on plastid DNA sequence data. *In* S. A. Ghazanfar AND H. J. Beentje [eds.], Taxonomy and ecology of African plants, their conservation and sustainable use, 73-83. Royal Botanic Gardens Kew, UK.

COOMES, D. A., AND P. J. GRUBB. 2003. Colonization, tolerance, competition and seed-size variation wihin functional groups. *Trends in Ecology and Evolution* 18: 283-291.

COPE, F. W. 1962. The mechanism of self-incompatibility in *Theobroma cacao* L. *Heredity* 17: 157-182.

COPLAND, B. J., AND R. J. WHELAN. 1989. Seasonal variation in flowering intensity and pollination limitation of fruit set in four co-occurring *Banksia species*. *Journal of Ecology* 77: 509-523.

COSTA, M. C. D., J. M. FARRANT, M. J. OLIVER, W. LIGTERINK, J. BUITINK, AND H. M. W. HILHORST. 2016. Key genes involved in desiccation tolerance and dormancy across life forms. *Plant Science* 251: 162–168.

COYNE, J. A., AND H. A. ORR. 2004. Speciation. Sinauer Assoc. Inc., Publ., Sunderland, MA, USA.

CRESSWELL, J. E., AND C. GALEN. 1991. Frequency-dependent selection and adaptive surfaces for floral character combinations: the pollination of *Polemonium viscosum*. *American Naturalist* 138: 1342-1353.

CRUZAN, M. B., P. R. NEAL, AND M. F. WILLSON. 1988. Floral display in *Phyla incisa*: consequences for male and female reproduction success. *Evolution* 42: 505-515.

DAFNI, A. 1984. Mimicry and deception in pollination. *Annual Review of Ecology and Systematics* 15: 259-278.

DAFNI, A. 1991. Advertisment, flower longevity, reward and nectar protection in Labiatae. *Acta Horticulturae* 288: 340-346.

------. 1992. Pollination ecology: a practical approach. Oxford University Press, Oxford, UK.

------. 1997. The response of *Amphicoma* spp. (Coleoptera; Glaphysidae) beetles to red models differing in area, shape, and symmetry. *Israeli Journal of Plant Sciences* 45: 247-254.

DAFNI, A., AND P. BERNHARDT. 1990. Pollination of terrestrial orchids of southern Australia and the Mediterranean region: systematic, ecological, and evolutionary implications. *Evolutionary Biology* 24: 193-252.

DAFNI, A., AND P. G. KEVAN. 1997. Flower size and shape: implications in pollination. *Israel Journal of Plant Sciences* 45: 201-212.

DAFNI, A., AND M. GIURFA. 1999. The functional ecology of floral guides in relation to insects behaviour and vision. *In* S. P. Wasser [ed.], Evolutionary Theory and Processes: Modern Prespectives, Papers in Honour of Eviatar Nevo, 363-383. Springer, Dordrecht.

DAFNI, A., M. LEHRER, AND P. G. KEYAN. 1997. Spatial flower parameters and insect spatial vision. *Biological Reviews* 72: 239-282.

DAHLGREN, R. M. T., H. T. CLIFFORD, AND P. F. YEO. 1985. The families of monocotyledons. Springer-Verlag, Berlin, Germany.

DALLING, J. W., AND S. P. HUBBELL. 2002. Seed size, growth rate and gap microsite conditions as determinants of recruitment success for pioneer species. *Journal of Ecology* 90: 557-568.

DANTHU, P., A. GUEYE, A. BOYE, D. BAUWENS, AND A. SARR. 2000. Seed storage behaviour of four Sahelian and Sudanian tree species (*Boscia senegalensis, Butyrospermum parkii, Cordyla pinnata* and *Saba senegalensis*). *Seed Science Research* 10: 183-187.

DARLINGTON, C. D., AND K. MATHER. 1949. The elements of genetics. Allen & Unwin, London, UK.

DARWIN, C. 1862a. On the two forms, or dimorphic condition, in the species of *Primula* and on their remarkable sexual relations. *Botanical Journal of the Linnean Society* 6: 77-96.

DARWIN, C. R. 1859. On the origin of species by means of natural selection or the preservation of favoured races in the struggle for life. John Murray, London, UK.

------. 1862b. On the various contrivances by which British and foreign orchids are fertilized by insects. Facsimile ed. John Murray, London, UK.

DARWIN, C. R. 1876. The Effects of Cross and Self Fertilization in the Vegetable Kingdom. John Murray, London, UK.

DARWIN, C. R. 1877. The various contrivances by which orchids are fertilised by insects. second edition, revised ed. John Murray, London, UK.

DAVIS, C. C., H. SCHAEFER, Z. XI, D. A. BAUM, M. J. DONOGHUE, AND L. J. HARMON. 2014. Long-term morphological stasis maintained by a plant-pollinator mutualism. *Proceedings of the National Academy of Sciences of the United States of America* 111: 5914-5919.

DAVIS, S. D., V. H. HEYWOOD, AND A. C. HAMILTON. 1994. Centres of Plant DIversity. A Guide and Strategy for their Conservation. IUCN Publications Unit, Cambridge, UK.

DAWS, M. I., N. C. GARWOOD, AND H. W. PRITCHARD. 2005. Traits of recalcitrant seeds in a semi-deciduous tropical forest in Panamá: some ecological implications. *Functional Ecology* 19: 874-885.

DE CASTRO, A., J. ARROYO, V. I. SIMÓN-PORCAR, AND M. HERRERO. 2015. Ovarian self-incompatibility in Narcissus papyraceus (Amaryllidaceae) is the result of a pre-zygotic response. *Botanical Journal of the Linnean Society* 177: 629-643.

DE CRAENE, L. R. 2018. Understanding the role of floral development in the evolution of angiosperm flowers: clarifications from a historical and physicodynamic perspective. *Journal of Plant Research* 131: 367-393.

DE JONG, T., AND P. KLINKHAMER. 2006. Evolutionary ecology of plant reproductive strategies. Cambridge University Press, Cambridge, UK.

DE LUCA, P. A., AND M. VALLEJO-MARIN. 2013. What's the 'buzz' about? The ecology and evolutionary significance of buzz-pollianation. *Current Opinion in Plant Biology* 16: 429-435.

DE NETTANCOURT, D. 1977. Incompatibility in Angiosperms. Springer-Verlag, Berlin.

------. 1997. Incompatibility in angiosperms. *Sexual Plant Reproduction* 10: 185-199.

------. 2001. Incompatibility and incongruity in wild and cultivated plants, Incompatibility and incongruity in wild and cultivated plants. Springer-Verlag, Berlin, Germany.

DE SMEDT, V., J. M. VAN HUYLENBROECK, AND P. C. DEBERGH. 1996. Influence of temperature and supplementary lighting on growth and flower initiation of *Clivia miniata* Regel. *Scientia Horticulturae* 65: 65-72.

DELL'OLIVO, A., M. E. HOBALLAH, T. GÜBITZ, AND C. KUHLEMEIER. 2011. Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution* 65: 1979–1991.

DELPH, L. F., AND C. M. LIVELY. 1989. The evolution of floral color change: pollinator attraction versus physiological constraints in *Fuchsia excorticata*. *Evolution* 43: 1252-1262.

DELPINO, F. 1867. Sugli apparecchi della fecondazione nelle piante antocarpee (fanerogame): sommario di osservazioni fatteneglianni 1865-1866. *Coi tipi di M. Cellini e C. alla Galileiana*.

DEVAUX, C., R. LANDE, AND E. PORCHER. 2014. Pollination ecology and inbreeding depression control individual flowering phenologies and mixed mating. *Evolution* 68: 3051-3065.

DEVAUX, C., E. PORCHER, AND R. LANDE. 2019. Mating systems and avoidance of inbreeding depression as evolutionary drivers of pollen limitation in animal-pollinated self-compatible plants. *Annals of Botany* 123: 327-336.

DINKEL, T., AND K. LUNAU. 2001. How drone flies (*Eristalis tenax* L., Syrphidae, Diptera) use floral guides to locate food sources. *Journal of Insect Physiology* 47: 1111-1118.

DOBSON, H. E. M. 1994. Floral volatiles in insect biology. *In* E. A. Bernays [ed.], Insect Plant Interactions, vol. 5, 47-81. CRC Press, Boca Raton, FL, USA.

DOBSON, H. E. M. 2006. Relationship between floral fragrance composition and type of pollinator. *In* N. Dudareva AND E. Pichersky [eds.], Biology of floral scents, 147-198. Taylor & Francis, Boca Raton, USA.

DOBSON, H. E. M., AND G. BERGSTRÖM. 2000. The ecology and evolution of pollen odor. *Plant Systematics and Evolution* 222: 63-87.

DOBSON, H. E. M., G. BERGSTRÖM, AND I. GROTH. 1990. Differences in fragrance chemistry between flower parts of *Rosa rugosa* Thunb. (Rosaceae). *Israel Journal of Botany* 39: 143-156.

DOBSON, H. E. M., I. GROTH, AND G. BERGSTROM. 1996. Pollen advertisement: chemical contrasts between whole-flower and pollen odors. *American Journal of Botany* 83: 877-885.

DODD, M. E., J. SILVERTOWN, AND M. W. CHASE. 1999. Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families. *Evolution* 53: 732-744.

DORNHAUS, A., AND L. CHITTKA. 1999. Insect behaviour: evolutionary origins of bee dances. *Nature* 401: 38-38.

DÖTTERL, S., U. GLÜCK, A. JÜRGENS, J. WOODRING, AND G. AAS. 2014. Floral reward, advertisement and attractiveness to honey bees in dioecious *Salix caprea*. *PLOS ONE* 9: e93421.

DULBERGER, R. 1964. Flower dimorphism and self-incompatibility in *Narcissus tazetta* L. *Evolution* 18: 361-363.

DUNCAN, G. 2004. Growing the miracle *Clivia*. Some tips on how to grow the newly discovered *Clivia mirabilis*. *Veld & flora.* 90: 148-149.

DUNCAN, G. D. 1999. Grow *Clivias*. 3rd ed. National Botanical Institute, Cape Town.

ECKERT, C. G., S. KALISZ, M. A. GEBER, R. SARGENT, E. ELLE, P. O. CHEPTOU, C. GOODWILLIE, et al. 2010. Plant mating systems in a changing world. *Trends in Ecology and Evolution* 25: 35-43.

EHRLICH, P. R., AND P. H. RAVEN. 1964. Butterflies and plants: a study in coevolution. *Evolution* 18: 586-608.

ELLIS, A. G., AND S. D. JOHNSON. 1999. Do pollinators determine hybridization patterns in sympatric *Satyrium* (Orchidaceae) species? *Plant Systematics and Evolution* 219: 137-150.

ELLIS, A. G., AND S. D. JOHNSON. 2009. The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *American Journal of Botany* 96: 793-801.

ERIKSSON, O., AND B. BREMER. 1992. Pollination systems, dispersal modes, life forms, and diversification rates in angiosperm families. *Evolution* 46: 258-266.

EVENARI, M. 1949. Germination inhibitors. *Botanical Review* 15: 153-194.

FAEGRI, K., AND L. VAN DER PIJL. 1979. The Principles of Pollination Ecology. Pergamon, Oxford.

FARNSWORTH, E. 2000. The ecology and physiology of viviparous and recalcitrant seeds. *Annual Review of Ecology and Systematics* 31: 107-138.

FENNER, M. 2000. Seeds: the ecology of regeneration in plant communities. 2nd ed. CABI Publishing, Wallingford, Oxon, UK.

FENSTER, C. B., AND M. R. DUDASH. 2001. Spatiotemporal variation in the role of hummingbirds as pollinators of *Silene virginica*. *Ecology* 82: 844-851.

FENSTER, C. B., AND S. MARTÉN-RODŔIGUEZ. 2007. Reproductive assurance and the evolution of pollination specialization. *International Journal of Plant Sciences* 168: 215-228.

FENSTER, C. B., W. S. ARMBRUSTER, P. WILSON, M. R. DUDASH, AND J. D. THOMSON. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology Evolution and Systematics* 35: 375-403.

FISHMAN, L. 2000. Pollen discounting and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution* 54: 1558-1565.

FISHMAN, L., AND J. H. WILLIS. 2008. Pollen limitation and natural selection on floral characters in the yellow monkeyflower, *Mimulus guttatus*. *New Phytologist* 177: 802-810.

FIZ-PALACIOS, O., H. SCHNEIDER, J. HEINRICHS, AND V. SAVOLAINEN. 2011. Diversification of land plants: insights from a family-level phylogenetic analysis. *BMC Evolutionary Biology* 11: 341.

FJELDSÅ, J., AND J. C. LOVETT. 1997. Geographical patterns of old and young species in African forest biota: the significance of specific montane areas as evolutionary centres. *Biodiversity and Conservation* 6: 325-346.

FORD, C. S., AND M. J. WILKINSON. 2012. Confocal observations of late-acting self-incompatibility in *Theobroma cacao* L. *Sexual Plant Reproduction* 25: 169-183.

FOREST, F., P. GOLDBLATT, J. C. MANNING, D. BAKER, J. F. COLVILLE, D. S. DEVEY, S. JOSE, et al. 2014. Pollinator shifts as triggers of speciation in painted petal irises (*Lapeirousia*: Iridaceae). *Annals of Botany* 113: 357-371.

FREY, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58: 2426-2437.

FRICKE, E. C., J. J. TEWKSBURY, AND H. S. ROGERS. 2014. Multiple natural enemies cause distance dependent mortality at the seed-to-seedling transition. *Ecology Letters* 17: 593-598.

GALEN, C. 1999. Flowers and enemies: predation by nectar-thieving ants in relation to variation in floral form of an alpine wildflower, *Polemonium viscosum*. *Oikos* 85: 426-434.

GALEN, C., AND M. E. A. NEWPORT. 1987. Bumble bee behavior and selection on flower size in sky pilot, *Polemonium viscosum*. *Oecologia* 74: 20-23.

GALEN, C., AND J. CUBA. 2001. Down the tube: Pollinators, predators, and the evolution of flower shape in the Alpine skypilot, *Polemonium viscosum*. *Evolution* 55: 1963-1971.

GALEN, C., AND B. BUTCHART. 2003. Ants in your plants: effects of nectar-thieves on pollen fertility and seed-siring capacity in the alpine wildflower, *Polemonium viscosum*. *Oikos* 101: 521-528.

GALLIOT, C., J. STUURMAN, AND C. KUHLEMEIER. 2006. The genetic dissection of floral pollination syndromes. *Current Opinion in Plant Biology* 9: 78-82.

GERITZ, S. A. H., E. VAN DER MEIJDEN, AND J. A. J. METZ. 1999. Evolutionary dynamics of seed size and seedling competitive ability. *Theoretical Population Biology* 55: 324-343.

GERMISHUIZEN, G., AND N. L. MEYER. 2003. Plants of southern Africa: an annotated checklist. *In* G. Germishuizen AND N. L. Meyer [eds.], Strelitzia 14, vol. 14. National Botanical Institute, Pretoria, South Africa.

GERVASI, D. D. L., AND F. P. SCHIESTL. 2017. Real-time divergent evolution in plants driven by pollinators. *Nature Communications* 8: 14691.

GIBBS, P. E. 2014. Late acting self incompatibility - the pariah breeding system in flowering plants. *New Phytologist* 203: 717-734.

GIBBS, P. E., AND M. BIANCHI. 1993. Post-pollination events in species of *Chorisia* (Bombacaceae) and *Tabebuia* (Bignoniaceae) with late-acting self-incompatibility. *Botanica Acta* 106: 64-71.

GIBBS, P. E., AND M. A. BIANCHI. 1999. Does Late-acting self-incompatibility (LSI) show family clustering? Two more species of Bignoniaceae with LSI: *Dolichandra cynanchoides* and *Tabebuia nodosa*. *Annals of Botany* 84: 449-457.

GIBBS, P. E., P. E. OLIVEIRA, AND M. B. BIANCHI. 1999. Post-zygotic control of selfing *Hymenaea stigonocarpa* (Leguminosae-Caesalpinioideae), a bat-pollinated tree of the Brazilian cerrados. *International Journal of Plant Sciences* 160: 72-78.

GIVNISH, T. J., AND A. SYTSMA. 1997. Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, UK.

GLOVER, B. J., C. A. AIROLDI, S. F. BROCKINGTON, M. FERNANDEZ-MAZUECOS, C. MARTINEZ-PEREZ, G. MELLERS, E. MOYROUD, AND L. TAYLOR. 2015. How have advances in comparative floral development influenced our understanding of floral evolution. *International Journal of Plant Sciences* 176: 307-323.

GOLDBLATT, P., AND J. C. MANNING. 1996. Phylogeny and speciation in *Lapeirousia* subgenus *Lapeirousia* (Iridaceae: Ixioideae). *Annals of the Missouri Botanical Garden* 83: 346-361.

GOLDBLATT, P., AND J. C. MANNING. 2006. Radiation of pollination systems in the iridaceae of sub-Saharan Africa. *Annals of Botany* 97: 317-344.

GOODWILLIE, C., S. KALISZ, AND C. G. ECKERT. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology Evolution and Systematics* 36: 47-79.

GOULSON, D., AND J. S. CORY. 1993. Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecological Entomology* 18: 315-320.

GOUWS, J. B. 1949. Karyology of some South African Amaryllidaceae. *Plant Life* 5: 54-60.

GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82-97.

GRANT, V. 1981. Plant Speciation. Columbia University Press, New York, USA.

GRANT, V., AND K. A. GRANT. 1965. Flower pollination in the Phlox family. Columbia University Press, New York, USA.

GRIBEL, R., AND P. E. GIBBS. 2002. High outbreeding as a consequence of selfed ovule mortality and single vector bat pollination in the Amazonian tree *Pseudobombax munguba* (Bombacaceae). *International Journal of Plant Sciences* 163: 1035-1043.

GROSS, K., M. SUN, AND F. P. SCHIESTL. 2016. Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orhcid. *PLOS ONE* 11: e0147975.

GROTEWOLD, E. 2006. The genetics and biochemistry of floral pigments. *Annual Review of Plant Biology* 57: 761-780.

GROTH, I., G. BERGSTROM, AND O. PELLMYR. 1987. Floral fragrances in *Cimicifuga*: chemical polymorphism and incipient speciation in *Cimicifuga simplex*. *Biochemical Systematics and Ecology* 15: 441-444.

GRUBB, P. J., AND D. J. METCALFE. 1996. Adaptation and inertia in the Australian tropical lowland rain-forest flora: contradictory trends in intergeneric and intrageneric comparisons of seed size in relation to light demand. *Functional Ecology* 10: 512-520.

GÜBITZ, T., M. E. HOBALLAH, A. DELL' OLIVO, AND C. KUHLEMEIR. 2009. *Petunia* as a model system for the genetics and evolution of pollination syndrome. *In* T. Gerats AND J. Strommer [eds.], Petunia, 29-49. Springer, New York, USA.

HAMILTON, K. N., C. A. OFFORD, P. CUNEO, AND M. A. DESEO. 2013. A comparative study of seed morphology in relation to desiccation tolerance and other physiological responses in 71 Eastern Australian rainforest species. *Plant Species Biology* 28: 51-62.

HANSEN, D. M., T. VAN DER NIET, AND S. D. JOHNSON. 2012. Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proceedings of the Royal Society B* 279: 634-639.

HAO, Y. Q., X. F. ZHAO, D. Y. SHE, B. XU, D. Y. ZHANG, AND W. J. LIAO. 2012. The role of late-acting self-incompatibility and early-acting inbreeding depression in governing female fertility in monkshood, *Aconitum kusnezoffii*. *PLOS ONE* 7: 1-7.

HARDER, L. D., AND J. D. THOMSON. 1989. Evolutionary options for maximizing pollen dispersal of animal-pollinated plants. *American Naturalist* 133: 323-344.

HARDER, L. D., AND S. C. H. BARRETT. 1995. Mating cost of large floral displays in hermaphrodite plants. *Nature* 373: 512-515.

HARDER, L. D., AND S. C. H. BARRETT. 1996. Pollen dispersal and mating patterns in animal-pollinated plants *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal-pollinated plants 140-190. Chapman & Hall, New York, USA.

HARDER, L. D., AND W. G. WILSON. 1998. A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. *American Naturalist* 152: 684-695.

HARDER, L. D., AND M. B. ROUTLEY. 2006. Pollen and ovule fates and reproductive performance by flowering plants. *In* L. D. Harder AND S. C. H. Barrett [eds.], Ecology and evolution of flowers 61-80. Oxford University Press, Oxford, UK.

HARDER, L. D., AND S. D. JOHNSON. 2008. Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Sciences* 169: 59-78.

------. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist* 183: 530-545.

HARDER, L. D., AND M. A. AIZEN. 2010. Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society Series B* 365: 529-543.

HARDER, L. D., N. M. WILLIAMS, C. Y. JORDAN, AND W. A. NELSON. 2001. The effects of floral design and display on pollinator economics and pollen dispersal. *In* L. Chittka AND J. D. Thomson [eds.], Cognitive ecology of pollination, 297-317. Cambridge University Press, Cambridge, UK.

HARGREAVES, A. L., S. D. JOHNSON, AND E. NOL. 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an "ornithophilous" African *Protea*. *Oecologia* 140: 295-301.

HARMS, K. E., S. J. WRIGHT, O. CALDERÓN, A. HERNÁNDEZ, AND E. A. HERRE. 2000. Pervasive density-dependent recruitment enhances seedling diversity in a tropical forest. *Nature* 404: 493.

HARPER, J. L., P. H. LOVELL, AND K. G. MOORE. 1970. The shapes and sizes of seeds. *Annual Review of Ecology and Systematics* 1: 327-356.

HEGLAND, S. J., A. NIELSEN, A. LA´ZARO, A.-L. BJERKNES, AND Ø. TOTLAND. 2009. How does climate warming affect plant-pollinator interactions? *Ecology Letters* 12: 184-195.

HERLIHY, C. R., AND C. G. ECKERT. 2002. Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* 416: 320-323.

HERMANN, K., AND C. KUHLEMEIR. 2011. The genetic architecture of natural variation in flower morphology. *Current Opinion in Plant Biology* 14: 60-65.

HERRERA, C. M. 1986. Vertebrate-dispersed plants: why they don't behave the way they should. *In* A. Estrada AND T. H. Fleming [eds.], Frugivores and Seed Dispersal, 5-18. Dr. W. Junk Publishers, Dordrecht.

------. 1989. Pollinator abundance, morphology, and flower visitation rate: analysis of the 'quantity' component in a plant-pollinator system. *Oecologia* 80: 241-248.

------. 1993. Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecological Monographs* 63: 251-275.

------. 1996. Floral traits and plant adaptation to insect pollinators: a devil's advocate approach. *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants, 65-87. Chapman & Hall, New York, USA.

HERRERA, C. M. 2000. Measuring the effects of pollinators and herbivores: evidence for non-additivity in a perennial herb. *Ecology* 81: 2170-2176.

HERRERA, C. M., AND P. JORDANO. 1981. *Prunus mahaleb* and birds: the high efficiency seed dispersal system of a temperate fruiting tree. *Ecological Monographs* 51: 203-218.

HERRERA, C. M., X. CERDA, M. B. GARCIA, J. GUITIAN, M. MEDRANO, P. J. REY, AND A. M. SANCHEZ-LAFUENTE. 2002. Floral integration, phenotypic covariance structure and pollinator variation in bumblebee-pollinated *Helleborus foetidus*. *Journal of Evolutionary Biology* 15: 108-121.

HERRERA, J. 1985. Nectar secretion patterns in southern Spanish Mediterranean scrublands. *Israel Journal of Botany* 34: 45-58.

HESLOP-HARRISON, J. 1983. Self-incompatibility: phenomenology and physiology. *Proceedings of the Royal Society of London, Series B* 218: 371-395.

HEY, J. 1992. Using phylogenetic trees to study speciation and extinction. *Evolution* 46: 627-640.

HILL, P. S. M., P. H. WELLS, AND H. WELLS. 1997. Spontaneous flower constancy and learning in honey bees as a function of colour. *Animal Behaviour* 54: 615-627.

HINATA, K., A. ISOGAI, AND K. ISUZUGAWA. 1994. Manipulation of sporophytic self-incompatibility in plant breeding. *In* E. G. Williams, A. E. Clarke, AND R. B. Knox [eds.], Genetic control of self-incompatibility and reproductive development in flowering plants, vol. Advances in cellular and molecular biology of plants, 102-115. Kluwer Academic Publishers Dordrecht, Netherlands.

HINGSTON, A. B., AND P. B. MCQUILLAN. 2000. Are pollination syndromes useful predictors of floral visitors in Tasmania? *Austral Ecology* 25: 600-609.

HIROTA, S. K., N. MIKI, A. A. YASUMOTO, AND T. YAHARA. 2019. UV bullseye contrast of *Hemerocallis* flowers attracts hawkmoths but not swallowtail butterflies. *Ecology and Evolution* 9: 52-64.

HOBALLAH, M., T. GUEBITZ, J. STUURMAN, L. BROGER, M. BARONE, T. MANDEL, A. DELL'OLIVO, AND C. KUHLEMEIER. 2007. Single gene-mediated shift in pollinator attraction in *Petunia*. *Plant Cell* 19: 779-790.

HOBALLAH, M. E., J. STUURMAN, T. C. J. TURLINGS, P. M. GUERIN, S. CONNETABLE, AND C. KUHLEMEIER. 2005. The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. *Planta* 222: 141-150.

HOKANSON, K. H., J. 2000. Early-acting inbreeding depression in three species of *Vaccinium* (Ericaceae). *Plant Sexual Reproduction* 13: 145-150.

HOLSINGER, K. E. 1996. Pollination biology and the evolution of mating systems in flowering plants. *Evolutionary Biology* 29: 107-149.

HONIBALL, C. B. 2000. Manipulation of flowering period and shoot manipulation in *Clivia miniata* (Lindley) Regel, [s.n.], Pretoria, South Africa.

HOULE, G. 1995. Seed dispersal and seedling recruitment: the missing link(s). *Ecoscience* 2: 238-244.

HOVE, A. A., S. J. MAZER, AND C. T. IVEY. 2016. Seed set variation in wild *Clarkia* populations: teasing apart the effects of seasonal resource depletion, pollen quality, and pollen quantity. *Ecology and Evolution* 6: 6524-6536.

HOWE, H. F. 1984. Constraints on the evolution of mutualisms. *American Naturalist* 123: 764-777.

HOWE, H. F. 1989. Scatter- and clump-dispersal and seedling demography: hypothesis and implications. *Oecologia* 79: 417-426.

HOWE, H. F. 1993. Specialized and generalized dispersal systems: where does 'the paradigm' stand? *Vegetatio* 107/108: 3-13.

HOWE, H. F., AND G. F. ESTABROOK. 1977. On intraspecific competition for avian dispersers in tropical trees. *American Naturalist* 111: 817-832.

HOWE, H. F., AND J. SMALLWOOD. 1982. Ecology of seed dispersal. *Annual Review of Ecology and Systematics* 13: 201-228.

HOWE, H. F., AND M. N. MIRITI. 2004. When seed dispersal matters. *BioScience* 54: 651-660.

HUBER, F. K., R. KAISER, W. SAUTER, AND F. P. SCHIESTL. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* 142: 564-575.

INARIYAMA, S. 1937. Karyotype studies in Amaryllidaceae. *Science reports of the Tokyo Bunrika Daigaku. Section B.* 3: 95-113.

IRWIN, R. E., S. Y. STRAUSS, S. STORZ, A. EMERSON, AND G. GUIBERT. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84: 1733-1743.

JAIN, S. K. 1976. The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* 7: 469-495.

JANZ, N. 2011. Ehrlich and Raven revisited: mechanisms underlying codiversification of plants and enemies. *Annual Review of Ecology and Systematics* 42: 71-89.

JANZEN, D. H. 1970. Herbivores and the number of tree species in tropical forests. *American Naturalist* 104: 501-528.

JANZEN, D. H., AND P. S. MARTIN. 1982. Neotropical anachronism: the fruits the gomphotheres ate. *Science* 215: 19-27.

JOËT, T., J.-M. OURCIVAL, M. CAPELLI, S. DUSSERT, AND X. MORIN. 2016. Explanatory ecological factors for the persistence of desiccation-sensitive seeds in transient soil seed banks: *Quercus ilex* as a case study. *Annals of Botany* 117: 165–176.

JOGESH, T., R. P. OVERSON, R. A. RAGUSO, AND K. A. SKOGEN. 2017. Herbivory as an important selective force in the evolution of floral traits and polliantor shifts *AoB PLANTS* 9: plw088.

JOHNSON, M. T. J., S. T. CAMPBELL, AND S. C. H. BARRETT. 2015. Evolutionary interactions between plant reproduction and defence against herbivores. *Annual Review of Ecology and Systematics* 46: 191-213.

JOHNSON, S. D. 1997. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. *Botanical Journal of the Linnean Society* 123: 225-235.

JOHNSON, S. D. 2006. Pollinator-driven speciation in plants. *In* L. D. Harder AND S. C. H. Barrett [eds.], The ecology and evolution of flowers 295-310. Oxford University Press, Oxford, UK.

------. 2010. The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society Series B* 365: 499-516.

JOHNSON, S. D., AND W. J. BOND. 1994. Red flowers and butterfly pollination in the fynbos of South Africa. *In* M. Arianoutsou AND R. H. Groves [eds.], Plant-Animal Interactions in Mediterranean-Type Ecosystems, 137-148. Kluwer Academic Publishers, Dordrecht, Netherlands.

JOHNSON, S. D., AND K. E. STEINER. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51: 45-53.

JOHNSON, S. D., AND K. E. STEINER. 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* 15: 190-193.

JOHNSON, S. D., AND K. E. STEINER. 2003. Specialized pollination systems in southern Africa. *South African Journal of Science* 99: 345-348.

JOHNSON, S. D., AND P. WESTER. 2017. Stefan Vogel's analysis of floral syndromes in the South African flora: An appraisal based on 60 years of pollination studies. *Flora* 232: 200-206.

JOHNSON, S. D., H. P. LINDER, AND K. E. STEINER. 1998. Phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). *American Journal of Botany* 85: 402-411.

JOHNSON, S. D., A. PAUW, AND J. MIDGLEY. 2001. Rodent pollination in the African lily *Massonia depressa* (*Hyacinthaceae*). *American Journal of Botany* 88: 1768-1773.

JOHNSON, S. D., H. C. BUTLER, AND A. ROBERTSON. 2019. Breeding systems in *Cyrtanthus* (Amaryllidaceae): variation in self-sterility and potential for ovule discounting. *Plant Biology* doi: 10.1111/plb.13025.

JOHNSTON, M. O., E. PORCHER, P.-O. CHEPTOU, C. G. ECKERT, E. ELLE, M. A. GEBER, AND E. AL. 2009. Correlations among fertility components can maintain mixed mating in plants. *American Naturalist* 173: 1-11.

JORDAAN, L. A., S. D. JOHNSON, AND C. T. DOWNS. 2011. The role of avian frugivores in germination of seeds of fleshy-fruited invasive alien plants. *Biological Invasions* 13: 1917-1930.

------. 2012. Wahlberg's epauletted fruit bat (*Epomophorus wahlbergi*) as a potential dispersal agent for fleshy-fruited invasive alien plants: effects of handling behaviour on seed germination. *Biological Invasions* 14: 959-968.

JORDANO, P., AND C. M. HERRERA. 1995. Shuffling the offspring: uncoupling and spatial discordance of multiple stages in vertebrate seed dispersal. *Ecoscience* 2: 230-237.

JORDANO, P., AND E. W. SCHUPP. 2000. Seed disperser effectiveness: the quantity component and patterns of seed rain for *Prunus mahaleb*. *Ecological Monographs* 70: 591-615.

JURGENS, A., T. WITT, AND G. GOTTSBERGER. 2003. Flower scent composition in *Dianthus* and *Saponaria* species (Caryophyllaceae) and its relevance for pollination biology and taxonomy. *Biochemical Systematics and Ecology* 31: 345-357.

JŰRGENS, A., S. L. WEE, A. SHUTTLEWORTH, AND S. D. JOHNSON. 2013. Chemical mimicry of insect oviposition sites: a global analysis of convergence in angiosperms *Ecology Letters* 16: 1157-1167.

KARIYAT, R. R., K. E. MAUCK, C. M. BALOGH, A. G. STEPHENSON, M. C. MESCHER, AND C. M. D. MORAES. 2013. Inbreeding in horsenettle (*Solanum carolinense*) alters night-time volatile emissions that guide oviposition by *Manduca sexta* moths. *Proceedings of the Royal Society of London, Series B* 280: 20130020.

KARRON, J. D., C. T. IVEY, R. J. MITCHELL, M. R. WHITEHEAD, R. PEAKALL, AND A. L. CASE. 2011. New perspectives on the evolution of plant mating systems. *Annals of Botany* 109: 493-503.

KAWAGOE, T., AND N. SUZUKI. 2005. Self-pollen on a stigma interferes with outcrossed seed production in a self-incompatible monoecious plant, *Akebia quinata* (Lardizabalaceae). *Functional Ecology* 19: 49-54.

KAY, K. M., P. A. REEVES, R. G. OLMSTEAD, AND D. W. SCHEMSKE. 2005. Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *American Journal of Botany* 92: 1899-1910.

KEASAR, T., Y. BILU, U. MOTRO, AND A. SHMIDA. 1997. Foraging choices of bumblebees on equally rewarding artificial flowers of different colors. *Israel Journal of Plant Sciences* 45: 223-233.

KELBER, A. 1997. Innate preferences for flower features in the hawkmoth *Macroglossum stellatarum*. *Journal of Experimental Biology* 200: 827-836.

KELBER, A., AND D. OSORIO. 2010. From spectral information to animal colour vision: experiments and concepts. *Proceedings of the Royal Society B: Biological Sciences* 277: 1617-1625.

KELBER, A., M. VOROBYEV, AND D. OSORIO. 2003. Animal colour vision - behavioral tests and physiological consepts. *Biological Reviews* 78: 81-118.

KENRICK, J., V. KAUL, AND E. G. WILLIAMS. 1986. Self-Incompatibility in *Acacia-Retinodes* : Site of pollen-tube arrest is the nucellus. *Planta* 169: 245-250.

KEVAN, P. G. 1972. Floral colors in the high arctic with reference to insect-flower relations and pollination. *Canadian Journal of Botany* 50: 2289-2316.

KIEPIEL, I., AND S. D. JOHNSON. 2014. Shift from bird to butterfly pollination in *Clivia* (Amaryllidaceae). *American Journal of Botany* 101: 190-200.

KLINKHAMER, P. G. L., AND T. J. DE JONG. 1990. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). *Oikos* 57: 399-405.

KLINKHAMER, P. G. L., T. J. DE JONG, AND G. J. DEBRUYN. 1989. Plant size and pollinator visitation in *Cynoglossum officinale*. *Oikos* 54: 201-204.

KLINKHAMER, P. G. L., T. J. DE JONG, AND R. A. WESSELINGH. 1991. Implications of differences between hermaphrodite and female flowers for attractiveness to pollinators and seed production. *Netherlands Journal of Zoology* 41: 130-143.

KNIGHT, R., AND H. H. ROGERS. 1955. Incompatibility in *Theobroma cacao*. *Heredity* 9: 66-77.

KNIGHT, T. M., J. A. STEETS, J. C. VAMOSI, S. J. MAZER, M. BURD, D. R. CAMPBELL, M. R. DUDASH, et al. 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology Evolution and Systematics* 36: 467-497.

KNUDSEN, J. T., AND L. TOLLSTEN. 1995. Floral scent in bat-pollinated plants: a case of convergent evolution. *Botanical Journal of the Linnean Society* 119: 45-57.

KNUDSEN, J. T., AND J. GERSHENZON. 2006. The chemical diversity of floral scent. *In* N. Dudareva AND E. Pichersky [eds.], Biology of floral scent, 27-52. Taylor & Francis, Boca Raton, USA.

KNUDSEN, J. T., L. TOLLSTEN, AND BERGSTRÖM. 1993. Floral scents: a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* 33: 253-280.

KNUTH, P. 1906. Handbook of Flower Pollination. Clarendon Press, Oxford, UK.

KOHN, J. R., AND S. C. H. BARRETT. 1994. Pollen discounting and the spread of a selfing variant in tristylous *Eichhornia paniculata*: evidence from experimental populations. *Evolution* 48: 1576-1594.

KÖLREUTER, J. G. 1761. Vorlauflge Nachricht von einigen das Geschlecht der Pflanzen betreffenden Versuchen und Beobachtung. Leipzig, Germany.

KOOPOWITZ, H. 1986. Conservation problems in the Amaryllidaceae. *Herbertia* 42: 21-25.

------. 2002. *Clivias*. Timber Press, Portland, USA.

KOSHITAKA, H., K. ARIKAWA, AND M. KINOSHITA. 2011. Intensity contrast as a crucial cue for butterfly landing. *Journal of Comparative Physiology A* 197: 1105-1112.

KRAUSS, S. L., R. D. PHILLIPS, J. D. KARRON, S. D. JOHNSON, D. G. ROBERTS, AND S. D. HOPPER 2017. Novel consequences of bird pollination for plant mating. *Trends in Plant Science* 22: 395-410.

KREBS, S. L., AND J. F. HANCOCK. 1990. Early-acting inbreeding depression and reproductive success in the highbush blueberry, *Vaccinium corymbosum* L. *Theoretical and Applied Genetics* 79: 825-832.

KREFT, H., AND W. JETZ. 2007. Global patterns and determinants of vascular plant diveristy. *Proceedings of the National Academy of Sciences of the United States of America* 104: 5925-5930.

LA ROSA, R. J., AND J. K. CONNER. 2017. Floral function: effects of traits on pollinators, male and female pollination success, and female fitness across three species of milkweeds (*Asclepias*). *American Journal of Botany* 104: 150-160.

LADOUX, T., AND E. A. FRIAR. 2006. Late-acting self-incompatibility in *Ipomopsis tenuifolia* (Gray) V. Grant (Polemoniaceae). *International Journal of Plant Sciences* 167: 463-471.

LAGOMARSINO, L. P., F. L. CONDAMINE, A. ANTONELLI, A. MULCH, AND C. C. DAVIS. 2016. The abiotic and biotic drivers of rapid diversification in Andean bellflowers (Campanulaceae). *New Phytologist* 210: 1430-1442.

LAMBERT, J. E. 2002. Exploring the link between animal frugivory and plant stratergies: the case of primate fruit processing and post-dispesal seed fate. *In* D. J. Levey, W. R. Silva, AND M. Galetti [eds.], Seed dispersal and frugivory: ecology, evolution and conservation. Third International Symposium-Workshop on Frugivores and Seed Dispersal, Sao Pedro, Brazil, 6-11 August 2000, 365-379. CABI Publishing, Wallingford, UK.

LAMONT, B. 1985. The significance of flower colour change in eight co-occurring shrub species. *Botanical Journal of the Linnean Society* 90: 145-155.

LANDE, R., AND D. W. SCHEMSKE. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39: 24-40.

LARSON, B. M. H., AND S. C. H. BARRETT. 2000. A comparative analyses of pollen limitation in flowering plants. *Biological Journal of the Linnean Society* 69: 503-520.

LAWSON, D. A., H. M. WHITNEY, AND S. A. RANDS. 2017. Nectar discovery speeds and multimodal displays: assessing nectar search times in bees with radiating and non-radiating guides. *Evolutionary Ecology* 31: 899-912.

LAYMAN, N. C., M. T. R. FERNANDO, C. R. HERLIHY, AND J. W. BUSCH. 2017. Costs of selfing prevent the spread of a selfcompatibility mutation that causes reproductive assurance. *Evolution* 71: 884-897.

LEISHMAN, M. R., AND M. WESTOBY. 1994. The role of large seed size in shaded conditions - experimental evidence. *Functional Ecology* 8: 205-214.

LEISHMAN, M. R., AND B. R. MURRAY. 2001. The relationship between seed size and abundance in plant communities model communities and observed patterns. *Oikos* 94: 151-161.

LEONARD, A. S., AND D. R. PAPAJ. 2011. 'X' marks the spot: The possible benefits of nectar guides to bees and plants. *Functional Ecology* 25: 1293-1301.

LEVEY, D. J. 1987. Seed size and fruit-handling techniques of avian frugivores. *American Naturalist* 129: 471-485.

LEVIN, D. A., AND H. W. KERSTER. 1974. Gene flow in seed plants. *Evolutionary Biology* 7: 139-220.

LEWIS, A. C. 1993. Learning and the evolution of resources: pollinators and flower morphology. *In* D. R. Papaj AND A. C. Lewis [eds.], Insect learning: ecology and evolutionary perspectives, 219-242. Chapman & Hall, London, UK.

LINDER, H. P. 2001. Plant diversity and endemism in Sub-Saharan tropical Africa. *Journal of Biogeography* 28: 169-182.

LIPOW, S. R., AND R. WYATT. 2000. Single gene control of post-zygotic self-incompatibiltiy in poke milkweed, *Asclepias exaltata* L *Genetics* 154: 893-907.

LLOYD, D. G. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. *American Naturalist* 113: 67-79.

LLOYD, D. G. 1992. Self-and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences* 153: 370-380.

LLOYD, D. G., AND M. S. WELLS. 1992. Reproductive biology of a primitive angiosperm, *Pseudowintera colorata* (Winteraceae), and the evolution of pollination systems in the Anthophyta. *Plant Systematics and Evolution* 181: 77-95.

LLOYD, D. G., AND D. J. SCHOEN. 1992. Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences* 153: 358-369.

LUNAU, K. 1992. Innate recognition of flowers by bumble bees: orientation of antennae to visual stamen signals. *Canadian Journal of Zoology* 70: 2139-2144.

LUNAU, K., G. FIESELMANN, B. HEUSCHEN, AND A. VAN DE LOO. 2006. Visual targeting of components of floral colour patterns in flower-naive bumblebees (*Bombus terrestris*; Apidae). *Naturewissenschaften* 93: 325-328.

MACIOR, L. W. 1982. Plant community and pollinator dynamics in the evolution of pollination mechanisms in *Pedicularis* (Scrophulariaceae). *In* J. A. Armstrong, J. M. Powell, AND A. J. Richards [eds.], Pollination and Evolution, 29-45. Royal Botanic Gardens, Sydney, Australia.

MAKINO, T. T., AND S. SAKAI. 2007. Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. *Functional Ecology* 21: 854-863.

MANNING, A. 1956. The effects of honey-guides. *Behaviour* 9: 114-139.

MANNING, J. 2005. Pollination in *Clivia In* R. Dixon, C. Felbert, AND J. van der Linde [eds.], *Clivia* Seven, 17-22. *Clivia* Society, Cape Town, South Africa.

MANNING, J. C., AND P. GOLDBLATT. 2005. Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae : Crocoideae) and the development of bimodal pollination strategies. *International Journal of Plant Sciences* 166: 459-474.

MANT, J., R. PEAKALL, AND F. P. SCHIESTL. 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? *Evolution* 59: 1449-1463.

MARQUES, A., G. BUIJS, W. LIGTERINK, AND H. HILHORST. 2018. Evolutionary ecophysiology of seed desiccation sensitivity. *Functional Plant Biology* 45: 1083-1095.

MARQUIS, R. J., D. SALAZAR, C. BAER, J. REINHARDT, G. PRIEST, AND K. BARNETT. 2016. Ode to Ehrlich and Raven or how herbivorous insects might drive plant speciation. *Ecology* 97: 2939-2951.

MARTÉN-RODRÍGUEZ, S., W. JOHN KRESS, E. J. TEMELES, AND E. MELÉNDEZ-ACKERMAN. 2011. Plant–pollinator interactions and floral convergence in two species of *Heliconia* from the Caribbean Islands. *Oecologia* 167: 1075-1083.

MATTHYSEN, E. 2012. Multicausality of dispersal: a review. *In* J. Clobert, M. Baguette, T. G. Benton, AND J. M. Bullock [eds.], Dispersal Ecology and Evolution, 3-18. Oxford University Press, Oxford, UK.

MCKAY, J. W. 1942. Self-sterility in the chinese chesnut (*Castanea mollissima*). *Proceedings of the American Society for Horticultural Science* 41: 156-160.

MCKEY, D. 1975. The ecology of coevolved seed dispersal systems. *In* L. E. Gilbert AND P. H. Raven [eds.], Coveolution of Animals and Plants, 159-191. University of Texas Press, TX, USA.

MEDEL, R., C. BOTTO-MAHAN, AND M. KALIN-ARROYO. 2003. Pollinator mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* 84: 1721-1732.

MEEROW, A. W., AND D. A. SNIJMAN. 1998. Amaryllidaceae. *In* K. Kubitzki [ed.], Families and genera of vascular plants vol. 3, 83-110. Springer Verlag, Berlin, Germany.

MEEROW, A. W., AND J. R. CLAYTON. 2004. Generic relationships among the baccate-fruited Amaryllidaceae (tribe Haemantheae) inferred from plastid and nuclear non-coding DNA sequences. *Plant Systematics and Evolution* 244: 141-155.

MEEROW, A. W., C. L. GUY, Q. LI, AND S. YANG. 2000. Phylogeny of the American Amaryllidaceae based on nrDNA ITS sequeneces. *Systematic Botany* 25: 708-726.

MEEROW, A. W., M. F. FAY, C. L. GUY, Q.-B. LI, F. Q. ZAMAN, AND M. W. CHASE. 1999. Systematics of Amaryllidaceae based on cladistic analysis of plastid sequence data. *American Journal of Botany* 86: 1325-1345.

MENZEL, R., AND A. SHMIDA. 1993. The ecology of flower colors and the natural colour vision of insect pollinators: the Israeli flora as a study case. *Biological Reviews* 68: 81-120.

METCALF, R. L., AND E. R. METCALF. 1992. Plant kairomones in insect ecology and control. Chapman & Hall, New York, USA.

MEYER, G. A., AND M. C. WITMER. 1998. Influene of seed processing by frugivorous birds on germination success of three North American shrubs. *American Midland Naturalist* 140: 129-139.

MIDDLETON, F. 2017. Pollinating agents of *Clivia mirabilis In* J. van Wyk [ed.], *Clivia Eighteen*. *Clivia* Society, Koedoespoort, South Africa.

MILLER, R. B. 1981. Hawkmoths and the geographic patterns of floral variation in *Aquilegia caerulea*. *Evolution* 35: 763-774.

MOELLER, D. A. 2005. Pollinator community structure and sources of spatial variation in plant-pollinator interactions in *Clarkia xantiana* ssp. *xantiana*. *Oecologia* 142: 28-37.

MOELLER, D. A., R. D. BRISCOE RUNQUIST, A. M. MOE, M. A. GEBER, C. GOODWILLIE, P.-O. CHEPTOU, C. G. ECKERT, et al. 2017. Global biogeography of mating system variation in seed plants. *Ecology Letters* 20: 375-384.

MOL, J., E. GROTEWOLD, AND R. KOES. 1998. How genes paint flowers and seeds. *Trends in Plant Science* 3: 212-217.

MOLES, A. T., AND M. WESTOBY. 2004. What do seedlings die from and what are the implications for evolution of seed size. *Oikos* 106: 193-199.

MØLLER, A. P., AND G. SORCI. 1998. Insect preference for symmetrical artificial flowers. *Oecologia* 114: 37-42.

MOORE, P. D. 2001. The guts of seeds dispersal. *Nature* 414: 406-407.

MORGAN, M. T., AND W. G. WILSON. 2005. Self-fertilization and the escape from pollen limitation in variable pollination environments. *Evolution* 59: 1143-1148.

MORGAN, M. T., W. G. WILSON, AND T. M. KNIGHT. 2005. Plant population dynamics, pollinator foraging, and the selection of self-fertilization. *American Naturalist* 166: 169-183.

MUCHHALA, N. 2007. Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *American Naturalist* 169: 494-504.

MÜLLER, H. 1883. The Fertilisation of Flowers [Translated and edited by D'Arcy W. Thompson]. Macmillan and Co., London, UK.

MUNOZ, F., C. VIOLLE, AND P. O. CHEPTOU. 2016. CSR ecological strategies and plant mating systems: outcrossing increases with competitiveness but stress-tolerance is related to mixed mating. *Oikos* 125: 1296-1303.

MURRAY, B. G., Y. RAN, P. J. DE LANGE, K. R. W. HAMMETT, J. T. TRUTER, AND Z. H. SWANEVELDER. 2004. A new species of *Clivia* (Amaryllidaceae) endemic to the Pondoland Centre of Endemism, South Africa. *Botanical Journal of the Linnean Society* 146: 369-374.

NAABORGH, A. T., AND M. T. M. WILLEMSE. 1991. The ovular incompatibility system in *Gasteria verrucosa* (Mill.) H. Duval. *Euphytica* 58: 231-240.

NATTERO, J., AND A. A. COCUCCI. 2007. Geographical variation in floral traits of the tree tobacco in relation to its hummingbird pollinator fauna. *Biological Journal of the Linnaean Society* 90: 657-667.

NAVARRO, L., G. AYENSA, V. FERRERO, AND J. M. SANCHEZ. 2012. The avoidance of self-interference in the endemic daffodil *Narcissus cyclamineus* (Amaryllidaceae). *Plant Ecology* 213: 1813-1822.

NEE, S. 2007. Inferring speciation rates from phylogenies. *Evolution* 55: 661-668.

NILSSON, L. A. 1988. The evolution of flowers with deep corolla tubes. *Nature* 334: 147-149.

NOSIL. 2012. Ecological Speciation. Oxford University Press, New York, USA.

OLLERTON, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. *Journal of Ecology* 84: 767-769.

OLLERTON, J., S. D. JOHNSON, AND A. B. HINGSTON. 2006. Geographical variation in diversity and specificity of pollination systems. *In* N. M. Waser AND J. Ollerton [eds.], Plant-pollinator interactions: from specialization to generalization, 283-308. Chicago University Press, Chicago, USA.

OLLERTON, J., S. D. JOHNSON, L. CRANMER, AND S. KELLIE. 2003. The pollination ecology of an assemblage of grassland asclepiads in South Africa. *Annals of Botany* 92: 807-834.

OLLERTON, J., A. R. RECH, N. M. WASER, AND D. PRICE. 2015. Using the literature to test pollination syndromes - some methodological cautions. *Journal of Pollination Ecology* 16: 119-125.

OLLERTON, J., A. KILLICK, E. LAMBORN, S. WATTS, AND M. WHISTON. 2007. Multiple meanings and modes: on the many ways to be a generalist flower. *Taxon* 56: 717-728.

OLLERTON, J., R. ALARCON, N. M. WASER, M. V. PRICE, S. WATTS, L. CRANMER, A. HINGSTON, et al. 2009. A global test of the pollinaiton syndrome hypothesis. *Annals of Botany* 103: 1471-1480.

OLLERTON, J., S. LIEDE-SCHUMANN, M. E. ENDRESS, U. MEVE, A. R. RECH, A. SHUTTLEWORTH, H. A. KELLER, et al. 2019. The diversity and evolution of pollination systems in large plant clades: Apocynaceae as a case study. *Annals of Botany* 123: 311-325.

ÔMURA, H., AND K. HONDA. 2005. Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* 142: 588-596.

ÔMURA, H., K. HONDA, AND N. HAYASHI. 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae,* to rape flowers. *Journal of Chemical Ecology* 25: 1895-1906.

PAMMENTER, N. W., AND P. BERJAK. 2000. Evolutionary and ecological aspects of recalcitrant seed biology. *Seed Science Research* 10: 301-306.

PANDEY, K. K. 1960. Evolution of gametophytic and sporophytic systems of self-incompatibility in angiosperms. *Evolution* 14: 98-115.

PANNELL, J. R., AND A.-M. LABOUCHE. 2013. The incidence and selection of multiple mating in plants. *Philosophical Transactions of the Royal Society* 368: 20120051.

PAROLO, G., T. ABELI, G. ROSSI, G. DOWGIALLO, AND D. MATTHIES. 2011. Biological flora of Central Europe: *Leucojum aestivum* L. *Perspectives in Plant Ecology, Evolution and Systematics* 13: 319-330.

PAUW, A. 2006. Floral syndromes accurately predict pollination by a specialized oil-collecting bee (*Rediviva peringueyi*, Melittidae) in a guild of South African orchids (Coryciinae). *American Journal of Botany* 93: 917-926.

PEÑA-EGAÑA, M., A. P. LOAYZA, AND F. A. SQUEO. 2018. Are pulp consumers effective seed dispersers? Tests with a large-seeded tropical relict tree. *Biotropica* 50: 898-907.

PENNY, J. H. J. 1983. Nectar guide color contrast: a possible relationship with pollination strategy. *New Phytologist* 95: 707-721.

PEREZ-BARRALES, R., P. VARGAS, AND J. ARROYO. 2006. New evidence for the Darwinian hypothesis of heterostyly: breeding systems and pollinators in Narcissus sect. Apodanthi. *New Phytologist* 171: 553-567.

PERRET, M., A. CHAUTEMS, R. SPICHIGER, T. G. BARRACLOUGH, AND V. SAVOLAINEN. 2007. The geographical pattern of speciation and floral diversification in the neotropics: The tribe Sinningieae (Gesneriaceae) as a case study. *Evolution; International Journal of Organic Evolution*: 1641-1660.

PETER, C. I., AND S. D. JOHNSON. 2013. A pollinator shift explains floral divergence in an orchid species complex in South Africa. *Annals of Botany* 113: 277-288.

PICHERSKY, E., AND D. R. GANG. 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. *Trends in Plant Science* 5: 439-445.

PLEASANTS, J. M., AND M. ZIMMERMAN. 1990. The effect of inflorescence size on pollinator visitation of *Delphinium nelsonii* and *Aconitum columbianum*. *Collectanea Botanica* 19: 21-39.

PORCHER, E., AND R. LANDE. 2005. The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biology* 18: 497-508.

PRITCHARD, H. W., M. I. DAWS, B. J. FLETCHER, C. S. GAMÉNÉ, H. P. MSANGA, AND W. OMONDI. 2004. Ecological correlates of seed desiccation tolerance in tropical African dryland trees. *American Journal of Botany* 91: 863-870.

PROCTOR, M., P. YEO, AND A. LACK. 1996. The Natural History of Pollination. Collins, London, UK.

RAGUSO, R. A 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology and Systematics* 39: 549-569.

------. 2001. Floral scent, olfaction, and scent-driven foraging behaviour. *In* L. Chittka AND J. D. Thomson [eds.], Cognitive ecology of pollination: animal behaviour and foral evolution, 83-105. Cambridge University Press, Cambridge, UK.

------. 2004. Why are some floral nectars scented? *Ecology* 85: 1486-1494.

RAGUSO, R. A., AND M. A. WILLIS. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. *Animal Behaviour* 64: 685-695.

------. 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, Manduca sexta. *Animal Behaviour* 69: 407-418.

RAGUSO, R. A., R. A. LEVIN, S. E. FOOSE, M. W. HOLMBERG, AND L. A. MCDADE. 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry* 63: 265-284.

RAMSEY, J., H. D. BRADSHAW, AND D. W. SCHEMSKE. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* 57: 1520-1534.

RAN, Y. 2000. Genetic analysis of the genus *Clivia*, Phd Thesis, University of Auckland, New Zealand.

RAN, Y., AND S. SIMPSON. 2005. In vitro propagation of the genus *Clivia*. *Plant Cell, Tissue and Organ Culture* 81: 239-242.

RAN, Y., B. G. MURRAY, AND K. R. W. HAMMETT. 1999. Karyotype analysis of the genus *Clivia* by Giemsa and fluorochrome banding and in situ hybridization. *Euphytica* 106: 139-147.

RAN, Y., B. G. MURRAY, AND K. R. HAMMETT. 2001. Evaluating genetic relationships between and within *Clivia* species using RAPDs. *Scientia Horticulturae* 90: 167-179.

RAN, Y., K. R. W. HAMMETT, AND B. G. MURRAY. 2001a. Hybrid Identification in *Clivia* (Amaryllidaceae) using Chromosome Banding and Genomic In Situ Hybridization. *Annals of Botany* 87: 457-462.

------. 2001b. Phylogenetic Analysis and Karyotype Evolution in the Genus *Clivia* (Amaryllidaceae). *Annals of botany.* 87: 823-830.

REES, M. 1996. Evolutionary ecology of seed dormancy and seed size. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences* 351: 1299-1308.

REES, M., AND M. WESTOBY. 1997. Game-theoretical evolution of seed mass in multi-species ecological models. *Oikos* 78: 116-126.

REY, P. J., C. M. HERRERA, J. GUITIAN, X. CERDA, A. M. SÁNCHEZ-LAFUENTE, M. MEDRANO, AND J. L. GARRIDO. 2006. The geographic mosaic in predispersal interactions and selection on *Helleborus foetidus* (Ranunculaceae). *Journal of Evolutionary Biology* 19: 21-34.

REYNOLDS, R. J., M. R. DUDASH, AND C. B. FENSTER. 2010. Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica Evolution* 64: 358-369.

REZNICK, D. N., AND R. E. RICKLEFS. 2009. Darwin's bridge between microevolution and macroevolution. *Nature* 457: 837-842.

RICKLEFS, R. E. 2004. A comprehensive framework for global patters in biodiveristy. *Ecology Letters* 7: 1-5.

------. 2006. Evolutionary diversification and the origin of the diversity-environment relationship. *Ecology* 87: S3-S13.

RIESEBERG, L. H., A. WIDMER, A. M. ARNTZ, AND J. M. BURKE. 2002. Directional selection is the primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences of the United States of America* 99: 12242-12245.

RITLAND, K. 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* 88: 221-228.

ROBERTS, E. H. 1973. Predicting the storage life of seeds. *Seed Science and Technology* 1: 499-514.

ROBERTSON, J. L., AND R. WYATT. 1990. Evidence for pollination ecotypes in the yellow-fringed orchid, *Platanthera ciliaris*. *Evolution* 44: 121-133.

ROSAS-GUERRERO, V., R. AGUILAR, S. MARTÉN-RODRÍGUEZ, L. ASHWORTH, M. LOPEZARAIZA-MIKEL, J. M. BASTIDA, AND M. QUESADA. 2014. A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecology Letters* 17: 233-400.

ROURKE, J. P. 2002. *Clivia mirabilis* (Amaryllidaceae: Haemantheae) a new species from Northern Cape, South Africa. *Bothalia* 32: 1-7.

ROURKE, J. P. 2003. Natural interspecific hybrids in *Clivia - C. miniata* x *C. caulescens* hybrids from Mpumalanga. *In* J. van der Linde, R. Dixon, AND C. Felbert [eds.], *Clivia Five*, *Clivia* Society, Cape Town, South Africa.

ROURKE, J. P. 2012. South African National Biodiversity Institute, Personal communication.

RUTHEFORD, M. C. 1997. Categorization of biomes. *In* R. M. Cowling, D. M. Richardson, AND S. M. Pierce [eds.], Vegeation of Southern Africa, 91-98. Cambridge University Press, Cambridge, UK.

SAGE, T. L., AND E. G. WILLIAMS. 1991. Self-incompatibility in *Asclepias*. *Plant Cell Incompatibility Newsletter* 23: 55-57.

SAGE, T. L., AND F. B. SAMPSON. 2003. Evidence for ovarian self-incompatibility as a cause of self-sterility in the relictual woody angiosperm, *Pseudowintera axillaris* (Winteraceae). *Annals of Botany* 91: 807-816.

SAGE, T. L., R. I. BERTIN, AND E. G. WILLIAMS. 1994. Ovarian and other late-acting self-incompatibility systems. *In* E. G. Williams, A. E. Clarke, AND R. B. Knox [eds.], Genetic control of self-Incompatibility and reproductive development in flowering plants, vol. 2, Advances in cellular and molecular biology, 116-140. Kluwer Academic Publishers, Dordrecht, Netherlands.

SAGE, T. L., V. PONTIERI, AND R. CHRISTOPHER. 2000. Incompatibility and mate recognition in monocotyledons. *In* K. L. Wilson AND D. A. Morrison [eds.], Monocots: Systematics and evolution, 270-276. CSIRO, Melbourne, Australia.

SAGE, T. L., B. C. HUSBAND, AND M. B. ROUTLEY. 2005. Plant breeding systems and pollen dispersal *In* A. Dafni, P. G. Kevan, AND B. C. Husband [eds.], Practical pollination biology. Enviroquest, Ontario, Canada.

SAGE, T. L., M. V. PRICE, AND N. M. WASER. 2006. Self-sterility in I*pomopsis aggregata* (Polemoniaceae) is due to prezygotic ovule degeneration. *American Journal of Botany* 93: 254-262.

SAGE, T. L., F. STRUMAS, W. W. COLE, AND S. C. H. BARRETT. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* 86: 855-870.

SALISBURY, E. J. 1942. The Reproductive Capacity of Plants. G. Bell and Sons, Ltd., London, UK.

SANDERSON, C. E., B. S. OROZCO, P. S. M. HILL, AND H. WELLS. 2006. Honeybee (*Apis mellifera ligustica*) response to differcences in handling time, rewards and flower colours. *Ethology* 112: 937-946.

SAPIR, Y., AND W. S. ARMBRUSTER. 2010. Pollinator-mediated selection and floral evolution: from pollination ecology to macroevolution. *New Phytologist* 188: 303-306.

SATO, D. 1938. Karyotype alteration and phylogeny. IV. Karyotypes in Amaryllidaceae with special reference to the SATchromosome. *Cytologia* 9: 203-242.

SCHAEFER, H. M., AND G. D. RUXTON. 2009. Deception in plants: mimicry or perceptual exploitation. *Trends in Ecology and Evolution* 24: 676-685.

SCHAEFER, H. M., V. SCHAEFER, AND D. J. LEVEY. 2004. How plant-animal interactions signal new insights in communication. *Trends in Ecology & Evolution* 19: 577-584.

SCHAFFER, W. M., AND M. V. SCHAFFER. 1979. The adaptive significance of variations in reproductive habit in the Agavaceae. II. Pollinator foraging behavior and selection for increased reproductive expenditure. *Ecology* 60: 1051-1069.

SCHEMSKE, D. W., AND H. D. BRADSHAW, JR. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus). Proceedings of the National Academy of Science, USA* 96: 11910-11915.

SCHEMSKE, D. W., AND P. BIERZYCHUDEK. 2001. Evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. *Evolution* 55: 1269-1282.

SCHIESTL, F. P. 2010. The evolution of floral scent and insect chemical communication. *Ecology Letters* 13: 643-656.

SCHIESTL, F. P., AND S. D. JOHNSON. 2013. Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution* 28: 307-315.

SCHIESTL, F. P., F. K. HUBER, AND J. M. GOMEZ. 2011. Phenotypic selection on floral scent: trade-off between attraction and deterrence? *Evolutionary Ecology* 25: 237-248.

SCHLUTER, D. 2000. The ecology of adaptive radiation. Oxford University Press, Oxford, UK.

SCHMID-HEMPEL, P., AND B. SPEISER. 1988. Effect of inflorescence size on pollination in *Epilobium angustifolium*. *Oikos* 53: 98-104.

SCHUPP, E. W. 1993. Quantity, quality and the effectiveness of seed dispersal by animals. *Vegetatio* 107/108: 15-29.

SCHUPP, E. W., AND M. FUENTES. 1995. Spatial patterns of seed dispersal and the unification of plant population ecology. *Ecoscience* 2: 267-275.

SCOBELL, S. A., AND P. E. SCOTT. 2002. Visitors and floral traits of a hummingbird-adapted cactus (*Echinocereus coccineus*) show only minor variation along an elevational gradient. *American Midland Naturalist* 147: 1-15.

SEARS, E. R. 1937. Cytological phenomena connected with self-sterility in the flowering plants. *Genetics* 22: 130-181.

SEAVEY, S. R., AND K. S. BAWA. 1986. Late-acting self-incompatibility in angiosperms. *Botanical Review* 52: 195-219.

SERRANO-SERRANO, M. L., J. ROLLAND, J. L. CLARK, N. SALAMIN, AND M. PERRET. 2017. Hummingbird pollinaiton and the diversification of angiosperms: an old and successful association in Gesneriaceae. *Proceedings of the Royal Society B*.

SHYKOFF, J. A., E. BUCHELI, AND O. KALTZ. 1997. Anther smut disease in *Dianthus silvester* (Caryophyllaceae): Natural selection on floral traits. *Evolution* 51: 383-392.

SILANDER, J. A. 1978. Density-dependent control of reproductive success in *Cassia biflora*. *Biotropica* 10: 292-296.

SIMMS, E. L., AND M. A. BUCHER. 1996. Pleiotropic effects of flower-color intensity on herbivore performance on *Ipomoea purpurea*. *Evolution* 50: 957-963.

SIMON, V. 2013. *Ecologia evolutiva del polimorfismo estilar en Narcissus papyraceus Ker-Gawl. (Amaryllidaceae)*. Ph.D. thesis, University of Seville, Spain.

SIMPSON, B. B., AND J. L. NEFF. 1983. Evolution and diversity of floral rewards. *In* C. E. Jones AND R. J. Little [eds.], Handbook of Experimental Pollination Biology, 142-159. Van Nostrand Reinhold, New York, USA.

SMITH, S. D., AND KRIEBEL. 2018. Convergent evolution of floral shape tied to pollinator shifts in Iochrominae (Solanaceae). *Evolution* 72: 688-697.

SMITH, S. D., C. ANE, AND D. A. BAUM. 2008. The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution* 62: 793-806.

SNIJMAN, D. A. 2003. Fire and distrobution of clivias in southern Africa. *Clivia* Five: 98-100. Pretoria, South africa.

SPAETHE, J., J. TAUTZ, AND L. CHITTKA. 2001. Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proceedings of the National Academy of Sciences of the United States of America* 98: 3898-3903.

SPECHT, C. D., AND D. G. HOWARTH. 2015. Adaptation in flower form: a comparative evodevo approach. *New Phytologist* 206: 74-90.

SPIES, P., AND J. J. SPIES. 2018. *Clivia* taxonomy revisited, using DNA barcode regions. *Acta Horticulturae* 1201: 503-514.

SPIES, P., J. P. GROBLER, AND J. J. SPIES. 2011. A review of phylogenetic relationships in the genus *Clivia*. *Philosophical Transactions in Genetics* 1: 168-207.

SPRENGEL, C. K. 1793. Discovery of the secret of nature in the strucure and fertilization of flowers. Reprinted, 1996. *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal-pollinated plants, 3-43. Chapman & Hall, London, UK.

STEBBINS, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307-326.

STEINER, K. E. 1989. The pollination of *Disperis* (Orchidaceae) by oil-collecting bees in southern Africa. *Lindleyana* 4: 164-183.

STRAUSS, G. L., AND J. B. WHITTALL. 2006. Non-pollinator agents of selection on floral traits. *In* L. D. Harder AND S. C. H. Barrett [eds.], The ecology and evolution of flowers, 120-138, Oxford: Oxford University Press, UK.

STREHER, N. S., E. GUERRA, R. LÜDTKE, J. SEMIR, AND J. H. A. DUTILH. 2018. Self-incompatibility in *Habranthus gracilifolius* (Amaryllidaceae): pre- and post-pollination barriers. *Brazilian Journal of Botany* 41: 375-384.

SUBBIAH, A., S. RAMDHANI, N. W. PAMMENTER, H. H. ANGUS, A. H. H. MACDONALD, AND S. E. NAIDOO. 2019. Towards understanding the incidence and evolutionary history of seed recalcitrance: An analytical review. *Perspectives in Plant Ecology, Evolution and Systematics* 37: 11-19.

SUN, M., K. GROSS, AND F. P. SCHIESTL. 2013. Floral adaptation to local pollinator guilds in a terrestrial orchid. *Annals of Botany* 113: 289-300.

SUN, S.-G., W. S. ARMBRUSTER, AND S.-Q. HUANG. 2016. Geographic consistency and variation in conflicting selection generated by pollinators and seed predators. *Annals of Botany* 118: 227-237.

SUTHERLAND, S. D., AND R. K. VICKERY, JR. 1993. On the relative importance of floral color, shape, and nectar rewards in attracting pollinators to *Mimulus*. *Great Basin Naturalist* 53: 107-117.

SWANEVELDER, D., AND R. C. FISHER. 2009. *Clivia*: Nature and nurture. Briza Publications, Pretoria, South Africa.

SWANEVELDER, Z. H. 2003. Diversity and population structure of *Clivia miniata* Lindl. (Amaryllidaceae): Evidence from molecular genetics and ecology. M.Sc. Dissertation. University of the Free State, Bloemfontein, South Africa.

SWANEVELDER, Z. H., A. E. VAN WYK, AND J. T. TRUTER. 2005. Amaryllidaceae. A new variety in the genus *Clivia*. *Bothalia* 35: 67-68.

SWANEVELDER, Z. H., J. T. TRUTER, AND A. E. VAN WYK. 2006. A natural hybrid in the genus *Clivia*. *Bothalia* 36: 77-80.

TERBORGH, J., N. PITMAN, M. SILMAN, H. SCHICHTER, AND P. V. NUNEZ. 2002. Maintenance of tree diversity in tropical forests. *In* D. J. Levey, W. R. Silva, AND M. Galetti [eds.], Seed dispersal and frugivory: ecology, evolution and conservation. CABI Publishing, New York, USA.

TEWKSBURY, J. J., G. P. NABHAN, D. NORMAN, H. SUZÁN, J. TUXILL, AND J. DONOVAN. 1999. *In situ* conservation of wild chilies and their biotic associates. *Conservation Biology* 13: 98-107.

THEIS, N., AND R. A. RAGUSO. 2005. The effect of pollination on floral fragrance in thistles. *Journal of Chemical Ecology* 31: 2581-2600.

THEIS, N., AND L. S. ADLER. 2012. Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. *Ecology* 93: 430-435.

THOMSON, J. D. 1988. Effects of variation in inflorescence size and floral rewards on the visitation rates of traplining pollinators of *Aralia hispida*. *Evolutionary Ecology* 2: 65-76.

TOTLAND, O. 2001. Environment-dependent pollen limitation and selection on floral traits in an alpine species. *Ecology* 82: 2233-2244.

TRIPP, E. A., AND P. S. MANOS. 2008. Is floral specialization and evolutionary dead end? Pollination system transitions in *Ruellia* (Acanthaceae). *Evolution; International Journal of Organic Evolution* 62: 1712-1737.

TWEDDLE, J. C., J. B. DICKIE, C. C. BASKIN, AND J. M. BASKIN. 2003. Ecological aspects of seed desiccation sensitivity. *Journal of Ecology* 91: 294-304.

UYENOYAMA, M. K., K. E. HOLSINGER, AND D. M. WALLER. 1993. Ecological and genetic factors directing the evolution of self-fertilization. *Oxford Surveys in Evolutionary Biology* 9: 327-381.

VALDIVIA, C. E., AND H. M. NIEMEYER. 2006. Do floral syndromes predict specialisation in plant pollination systems? Assessment of diurnal and nocturnal pollination of *Escallonia myrtoidea*. *New Zealand Journal of Botany* 44: 135-141.

VALENTE, L. M., J. C. MANNING, P. GOLDBLATT, AND P. VARGAS. 2012. Did pollination shift drive diversification in southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *American Naturalist* 180: 83-98.

VALLEJO-MARÍN, M., M. E. DORKEN, AND S. C. H. BARRETT. 2010. The ecological and evolutionary consequences of clonality for plant mating. *Annual Review of Ecology and Systematics* 41: 193-213.

VALTUENA, F. J., T. RODRIGUEZ-RIANO, F. ESPINOSA, AND A. ORTEGA-OLIVENCIA. 2010. Self-sterility in two *Cystis* species (Leguminosae, Papilionoideae) due to early-acting inbreeding depression. *American Journal of Botany* 97: 123-135.

VAN DER MERWE, L., H. ROBBERTSE, AND B. DE KOCK. 2005. Cultivation of *Clivias*. *Clivia* Society, Pretoria, South Africa.

VAN DER NIET, T., AND S. D. JOHNSON. 2009. Patterns of plant speciation in the Cape floristic region. *Molecular Phylogenetics and Evolution* 51: 85-93.

VAN DER NIET, T., AND S. D. JOHNSON. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* 27: 353-361.

VAN DER NIET, T., R. PEAKALL, AND S. D. JOHNSON. 2014. Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany* 113: 199-211.

VAN DER PIJL, L. 1969. Evolutionary action of tropical animals on the reproduction of plants. *Biological Journal of the Linnean Society* 1: 85-96.

------. 1982. Principles of Dispersal in Higher Plants, 154 pp. Springer-Verlag, Berlin, Germany.

VAN KLEUNEN, M., AND S. D. JOHNSON. 2005. Testing for ecological and genetic allee effects in the invasive shrub *Senna didymobotrya* (Fabaceae). *American Journal of Botany* 92: 1124-1130.

VAN WYK, A. E. 1994. Maputaland-Pondoland Region. *In* S. D. Davis, V. H. Heywood, AND A. C. Hamilton [eds.], Centres of plant diversity. A guide and strategy for their conservation., Volume 1: pp. 227-235. IUCN Publications Unit Cambridge, UK.

VAN WYK, A. E., AND G. F. SMITH. 2001. Regions of floristic endemism in Southern Africa. A review with emphasis on succulents Umdaus Press, Pretoria, South Africa.

VANDER WALL, S. B., P.-M. FORGET, J. E. LAMBERT, AND P. E. HULME. 2005. Seed fate pathways: filling the gap between parent and offspring. *In* P.-M. Forget, J. E. Lambert, P. E. Hulme, AND S. B. Vander Wall [eds.], Seed fate: predation, dispersal and seedling establishment. CAB International, Wallingford, UK.

VAUGHTON, G., AND M. RAMSEY. 2010. Floral emasculation reveals pollen quality limitaion of seed output in *Bulbine bulbosa* (Asphodelaceae). *American Journal of Botany* 91: 174-178.

VAUGHTON, G., M. RAMSEY, AND S. D. JOHNSON. 2010. Pollination and late-acting self-incompatibility in *Cyrtanthus breviflorus* (Amaryllidaceae): implications for seed production. *Annals of Botany* 106: 547-555.

VEREECKEN, N. J., AND F. P. SCHIESTL. 2008. The evolution of imperfect floral mimicry. *Proceedings of the National Academy of Sciences of the USA* 105: 7484-7488.

VOGEL, S. 1954. Blütenbiologische Typen als Elemente der Sippengliederug, dargestellt anhand der Flora Südafrikas. Fischer, Jena, South Africa.

------. 1983. Ecophysiology of zoophilic pollination. *In* O. L. Lange, P. S. Nobel, C. B. Osmond, AND H. Ziegler [eds.], Physiological plant ecology, 560-611. Springer Verlag, Berlin, Germany.

VOGLER, D. W., AND S. KALISZ. 2001. Sex among the flowers: The distribution of plant mating systems. *Evolution* 55: 202-204.

WALTERS, C., P. BERJAK, N. W. PAMMENTER, K. KENNEDY, AND P. RAVEN. 2013. Preservation of recalcitrant seeds. *Science* 339: 915-916.

WANG, B. C., AND T. B. SMITH. 2002. Closing the seed dispersal loop. *Trends in Ecology and Evolution* 17: 379-385.

WANG, C., AND J. LEI. 2012. *In vitro* induction of tetraploids from immature embryos through colchicine treatments in *Clivia miniata* Regel. *African Journal of Agricultural Research* 7: 3712-3718.

WANG, J., Y. A. EL-KASSABY, AND K. RITLAND. 2012. Estimating selfing rates from reconstructed pedigrees using multi-locus genotype data. *Molecular Ecology* 21: 100-116.

WARD, M., AND S. D. JOHNSON. 2005. Pollen limitation and demographic structure in small fragmented populations of *Brunsvigia radulosa* (Amaryllidaceae). *Oikos* 108: 253-262.

WARREN, J., AND S. MACKENZIE. 2001. Why are all color combinations not equally represented in as flower-color polymorphisms? *New Phytologist* 151: 237-241.

WASER, N. M. 1986. Flower constancy: definition, cause, and measurement. *American Naturalist* 127: 593-603.

WASER, N. M. 2006. Specialization and generalization in plant-pollinator interactions: a historical perspective. *In* N. M. Waser AND J. Ollerton [eds.], Plant-pollinator interactions: from specialization to generalization, 3-17. University of Chicago Press, Chicago and London.

WASER, N. M., AND M. V. PRICE. 1983. Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302: 422-424.

WASER, N. M., AND M. V. PRICE. 1985. The effect of nectar guides on pollinator preference: experimental studies with a montane herb. *Oecologia* 67: 121-126.

WASER, N. M., AND M. V. PRICE. 1991. Reproductive costs of self-pollination in *Ipomopsis aggregata* (Polemoniaceae): are ovules usurped? *American Journal of Botany* 78: 1036-1043.

WASER, N. M., AND D. R. CAMPBELL. 2004. Ecological speciation in flowering plants. *In* U. Dieckmann, M. Doebeli, M. J. Metz, AND D. Tautz [eds.], Adaptive speciation, 264-277. Cambridge University Press, Cambridge, UK.

WASER, N. M., L. CHITTKA, M. V. PRICE, N. M. WILLIAMS, AND J. OLLERTON. 1996. Generalization in pollination systems, and why it matters. *Ecology* 77: 1043-1060.

WEISS, M. R. 1995. Floral color change: a widespread functional convergence. *American Journal of Botany* 82: 167-185.

WELLER, S. G., AND A. K. SAKAI. 1999. Using phylogenetic approaches for analysis of plant breeding system evolution. *Annual Review of Ecology and Systematics* 30: 167-199.

WENNY, D. G. 2001. Advantages of seed disperasal: A re-evaluation of directed dispersal. *Evolutionary Ecology Research* 3: 51-74.

WERNER, P. A. 1976. Ecology of plant populations in successional environments. *Systematic Botany* 1: 246-268.

WESTOBY, M., M. LEISHMAN, AND J. LORD. 1996. Comparative ecology of seed size and dispersal. *Philosophical Transactions of the Royal Society of London, Series B, Biological Science* 351: 1309-1317.

WHEELWRIGHT, N. T., AND G. H. ORIANS. 1982. Seed dispersal by animals: contrasts with pollen dispersal, problems of terminology, and constraints on coevolution. *American Naturalist* 119: 402-413.

WHITEHEAD, M. R., R. LANFEAR, R. J. MITCHELL, AND J. D. KARRON. 2018. Plant mating systems often vary widely among populations. *Frontiers in Ecology and Evolution* 6: 38.

WHITEHOUSE, H. L. K. 1951. Multiple-allelomorph incompatibility of pollen and style in the evolution of the angiosperms. *Annals of Botany New Series* 14: 198-216.

WHITNEY, H. M., AND B. J. GLOVER. 2007. Morphology and development of floral features recognised by pollinators. *Arthropod-Plant Interactions* 1: 147-158.

WHITTALL, J. B., AND S. A. HODGES. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature.* 477: 706-709.

WHITTALL, J. B., C. VOELCKEL, D. J. KLIEBENSTEIN, AND S. A. HODGES. 2006. Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in *Aquilegia*. *Molecular Ecology* 15: 4645-4657.

WHITTEN, W. M., AND N. H. WILLIAMS. 1992. Floral fragrances of *Stanhopea* (Orchidaceae). *Lindleyana* 7: 130-153.

WILLS, C., R. CONDIT, R. B. FOSTER, AND S. P. HUBBELL. 1997. Strong density- and diversity-related effects help to maintain a tree species diversity in a neotropical forest. *Proceedings of the National Academy of Sciences of the United States of America* 94: 1252-1257.

WILSON, P., M. C. CASTELLANOS, J. N. HOGUE, J. D. THOMSON, AND W. S. ARMBRUSTER. 2004. A multivariate search for pollination syndromes among penstemons. *Oikos* 104: 345-361.

WINTER, J. 2000. The natural distribution and ecology of *Clivia*. *In* M. Dower, C. Felbert, AND J. Winter [eds.], *Clivia* Yearbook 2, vol. 2, 5-9. Kirstenbosch National Botanical Gardens, Cape Town, South Africa.

WITMER, M. C., AND A. S. CHEKE. 1991. The dodo and the tambalacoque tree: an obligate mutualism reconsidered. *Oikos* 61: 133-137.

WYSE, S. V., AND J. B. DICKIE. 2017. Predicting the global incidence of seed desiccation sensitivity. *Journal of Ecology* 105: 1082-1093.

XU, Z., AND L. CHANG. 2017. Amaryllidaceae, identification and control of common weeds: Volume 3, Springer Singapore, Singapore.

ZANG, C., N. J. VEREECKEN, L. WANG, B. TIAN, A. DAFNI, Y. YANG, AND Y. DUAN. 2017. Are nectar guide colour changes a reliable signal to pollinators that enhance reproductive success? *Plant Ecology & Diversity* 10 89-96.

ZHANG, L., S. C. H. BARRETT, J. Y. GAO, J. CHEN, W. W. COLE, Y. LIU, Z. L. BAI, AND Q. J. LI. 2005. Predicting mating patterns from pollination syndromes: The case of "sapromyiophily" in *Tacca chantrieri* (Taccaceae). *American Journal of Botany* 92: 517-524.

ZHU, K., C. W. WOODALL, J. V. D. MONTEIRO, AND J. S. CLARK. 2015. Prevalence and strength of density-dependent tree recruitment. *Ecology* 96: 2319-2327.

CHAPTER 2

SHIFT FROM BIRD TO BUTTERFLY POLLINATION IN *CLIVIA* (AMARYLLIDACEAE)

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SHIFT FROM BIRD TO BUTTERFLY POLLINATION IN *CLIVIA* (AMARYLLIDACEAE)[1]

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- *Premise of the study:* Pollinator shifts have been implicated as a driver of divergence in angiosperms. We tested the hypothesis that there was a transition from bird- to butterfly pollination in the African genus *Clivia* (Amaryllidaceae) and investigated how floral traits may have been either modified or retained during this transition.
- *Methods:* We identified pollinators using field observations, correlations between lepidopteran wing scales and pollen on stigmas, and single-visit and selective exclusion experiments. We also quantified floral rewards and advertising traits.
- *Key results:* The upright trumpet-shaped flowers of *C. miniata* were found to be pollinated effectively by swallowtail butterflies during both nectar-feeding and brush visits. These butterflies transfer pollen on their wings, as evidenced by positive correlations between wing scales and pollen loads on stigmas. All other *Clivia* species have narrow pendulous flowers that are visited by sunbirds. Selective exclusion of birds and large butterflies from flowers of two *Clivia* species resulted in a significant decline in seed production.
- *Conclusions:* From the distribution of pollination systems on available phylogenies, it is apparent that a shift took place from bird- to butterfly pollination in *Clivia*. This shift was accompanied by the evolution of trumpet-shaped flowers, smaller nectar volume, and emission of scent, while flower color and nectar chemistry do not appear to have been substantially modified. These results are consistent with the idea that pollinator shifts can explain major floral modifications during plant diversification.

Key words: Bird pollination; butterfly pollination; floral scent; nectar chemistry; pollen; pollination syndrome; pollinator shift; scent; spectral reflectance.

Shifts between pollinators are often associated with evolutionary modification of floral traits and are thought to be a major driver of diversification in angiosperms (Stebbins, 1970; Harder and Johnson, 2009; van der Niet and Johnson, 2012). Other macroevolutionary evidence for the role of pollinators in shaping floral form is that unrelated taxa pollinated by similar pollinator assemblages often display convergent suites of floral traits known as floral syndromes (Faegri and van der Pijl, 1979; Fenster et al., 2004). The shifts that produce these patterns of diversification within clades, and convergence among them, are believed to be influenced by availability, behavior, and per-visit effectiveness of pollinators at the local population level (Stebbins, 1970), as well as intrinsic evolutionary constraints and opportunities (Stebbin's "lines of least resistance"). There is still much that is unknown about which trait modifications are critical for inducing pollinator shifts (Schemske and Bradshaw, 1999; Shuttleworth and Johnson, 2010), and whether shifts are more likely to occur between pollination systems that share many traits in common than between pollination systems that have few traits in common (Aigner, 2001). To address these questions, it is necessary to have data not only on the identity and effectiveness of pollinators, but also the floral reward and advertising traits of plant species with known phylogenetic relationships (van der Niet and Johnson, 2012).

Studies of several plant lineages have provided evidence for shifts between bird and lepidopteran pollination (Perret et al., 2007; Whittall and Hodges, 2007; Tripp and Manos, 2008; Gübitz et al., 2009). The shared utility of long corolla tubes in both bird and lepidopteran pollination systems may facilitate these shifts. In *Aquilegia*, for example, hawkmoth pollination evolved from hummingbird pollination (Whittall and Hodges, 2007), while in *Petunia* there is at least one case of hummingbird pollination evolving from hawkmoth-pollinated ancestors (Gübitz et al., 2009). A recent literature survey (van der Niet and Johnson, 2012) indicated that shifts involving butterfly pollination are poorly documented relative to those involving other Lepidoptera. Studies of the irid genera *Gladiolus* and *Tritoniopsis* indicate that butterfly pollination in these lineages evolved mostly from long-tongued bee and long-proboscid fly pollination systems (Manning and Goldblatt, 2005; Valente et al., 2012). Although not previously documented to our knowledge, shifts between bird and butterfly pollination systems seem plausible because of their shared associations with unscented tubular red or orange flowers (Faegri and van der Pijl, 1979).

Clivia (Amaryllidaceae) is one of the best-known ornamental plant groups worldwide (Koopowitz, 2002), yet the reproductive biology of *Clivia* species has not previously been investigated in the wild. Four of the species (*C. nobilis* Lindl., *C. gardenii* Hook., *C. caulescens* R. A. Dyer, and *C. mirabilis* Rourke) have tubular-pendulous flowers that conform to the syndrome of bird-pollination, while a fifth species *C. miniata* (Lindl.) Bosse has upright, trumpet-shaped flowers consistent with the syndrome of butterfly pollination (Manning, 2005). Phylogenetic studies of *Clivia* suggest that *C. miniata*, with its unique trumpet-shaped flowers, occupies a relatively derived position, sister to that of the *C. gardenii* species complex, with the remaining pendulous flowered species occupying more basal lineages (Conrad, 2008) (Fig. 1). It was therefore hypothesized that a shift took place from sunbird- to

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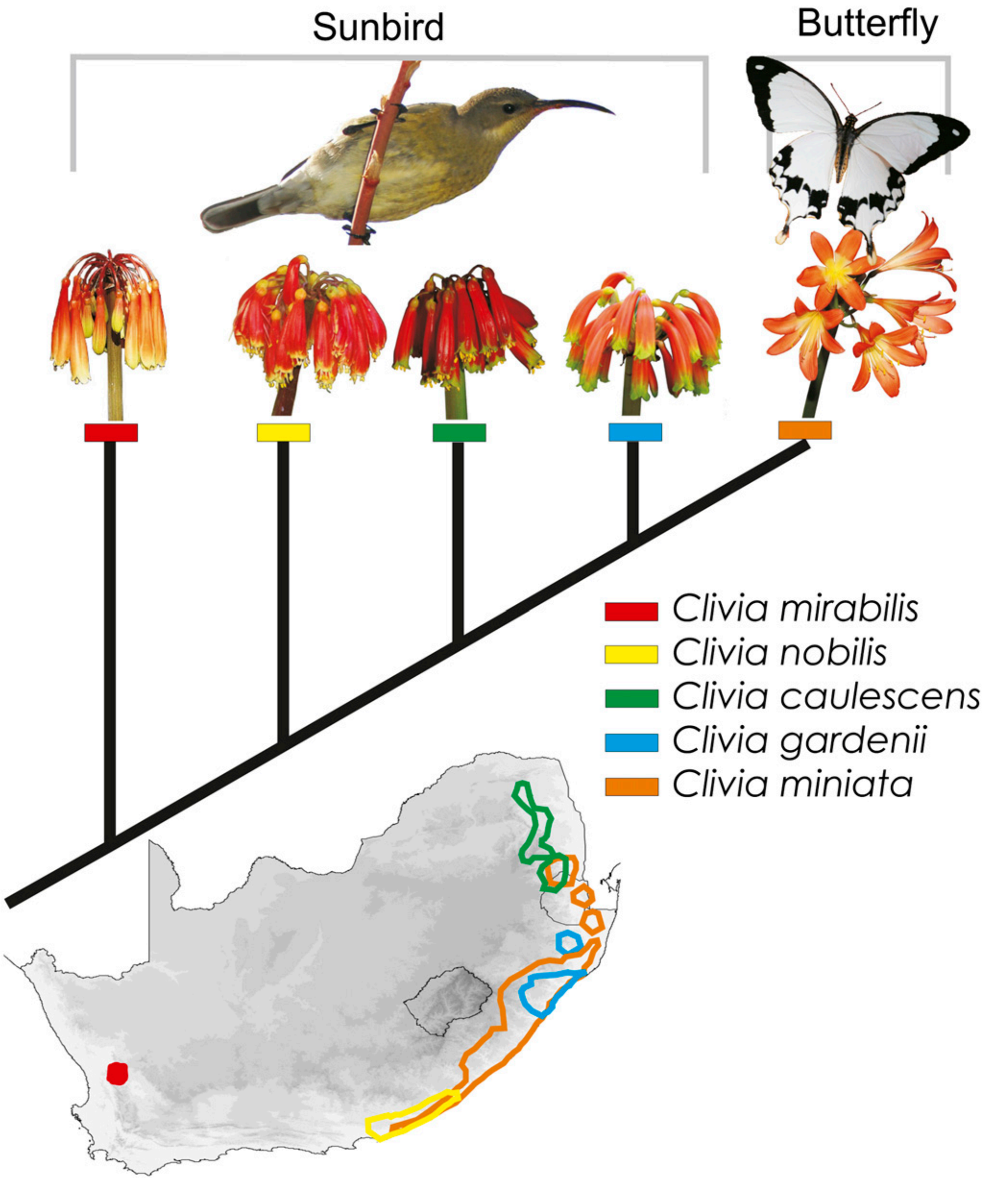

Fig. 1. Distribution of *Clivia* species in southern Africa and their phylogenetic relationships according to Conrad (2008). Butterfly pollination in *C. miniata* represents a unique and most recent evolutionary modification in the genus, while bird pollination represents the ancestral condition. Inflorescence and pollinator images are not to the same scale.

butterfly pollination in the genus. The aims of this study were (1) to determine whether pendulous and trumpet-flowered *Clivia* species are, as hypothesized, pollinated by sunbirds and butterflies, respectively; and, (2) to determine whether floral traits such as morphology, spectral reflectance, nectar and scent emissions were modified during the putative pollinator shift.

MATERIALS AND METHODS

Species and Study sites—*Clivia* Lindl. (Amaryllidaceae) is a horticulturally important taxon of long-lived evergreen perennials within the African tribe Haemantheae (Meerow et al., 1999; Rourke, 2002). The genus consists of five species that are endemic to southern Africa. With the exception of the semixeric *C. mirabilis*, which is adapted to a Mediterranean type climate (Rourke, 2002), *Clivia* species do not tolerate full sun and favor cool, shady, moist, coastal, and Afromontane forest habitats (Swanevelder, 2003). Although horticulturists sometimes succeed in producing *Clivia* seeds from hand-selfing (Swanevelder and Fisher, 2009), our studies indicate that *Clivia* species have a late-acting self-incompatibility system, and are therefore fully reliant on pollinator visits for seed production (I. Kiepiel, unpublished data). *Clivia* flowers are protogynous and remain open for up to three weeks if not pollinated.

This study focuses on *C. miniata*, the only member of the genus with upright trumpet-shaped flowers, and *C. gardenii*, a pendulous flowered form. The two species have a similar habitat and geographical distribution, overlapping along the eastern coast of South Africa (Fig. 1). Accordingly, any differences in their pollinator spectrum should reflect interactions between pollinators and floral features, rather than geographical differences in pollinator distribution. *Clivia robusta* B. G. Murray, Ran, de Lange, Hammett, Truter, Swanev. (Murray et al., 2004) has been distinguished from other forms of *C. gardenii* by the large size of plants, their swamp habitat, anther exsertion, and unusual karyology (Murray et al., 2004), but this taxonomic arrangement is controversial because of the absence of any consistent DNA sequence differences or quantitative studies of morphological discontinuities in wild populations. We have, therefore. followed local taxonomic authorities (Conrad, 2008; Rourke, 2012) in treating *C. robusta* as a "robust" form of *C. gardenii.*

The taxa examined in this paper are listed as vulnerable (Raimondo et al., 2009); consequently we do not provide detailed plant localities, beyond the general area, reserve, or district in which they occur. Details of localities are given on plant vouchers deposited in the Bews Herbarium (NU), University of KwaZulu-Natal, Pietermaritzburg (NU0038187; NU0038188; NU0038189; NU0038190; NU0038191). We studied five populations of the "robust" form of *C. gardenii* in the Pondoland Centre of Endemism (PC) north of the Umtamvuna Nature Reserve (UNR) in southern KwaZulu-Natal, South Africa. In addition, two populations of the "gracile" form of *C. gardenii* were observed at Gibba Gorge near Pinetown, KwaZulu-Natal. Seven *C. miniata* populations were studied, two of which were situated in the UNR, three in the Afromontane forests of the Karkloof mountains in the KwaZulu-Natal Midlands, and two on the outskirts of Pietermaritzburg, KwaZulu-Natal.

Pollinator observations—Field observations of *C. miniata* and *C. gardenii* were undertaken throughout their flowering periods of August to November (austral spring to early summer) and late March to early August (austral autumn to winter), respectively, during 2007–2012. In total, over 400 hours of observation were logged for *C. miniata*, 50 of which were nocturnal observations made throughout all periods of the night. Over 220 hours of observation were logged for *C. gardenii*, of which over 20 hours were night observations, made primarily after dusk and prior to dawn. Pollinators and other flower visitors were identified and recorded, together with their foraging behavior and flight patterns. Butterfly visitation data obtained for *C. miniata* at the Karkloof and UNR forests were obtained primarily over a period of two weeks for each site in the flowering seasons of 2010 and 2008, respectively, with some additional observations of butterfly behavior at the UNR site over six days in 2013. For individual butterfly visits to *C. miniata* flowers, we recorded whether they brushed past or alighted on a flower, and whether anther and stigma contact were made by the butterfly in the course of the visit. In addition, the number of flowers probed per plant were recorded, as well as the duration of visits and nectar probing. These data were recorded for all (126) of the butterfly visits recorded at the Karkloof site in 2010, and for 89 of the 143 visits recorded at the UNR site in 2008. Additional data on the proportion of visits that involve either flower brushing or alighting were recorded for 62 visits by *Papilio dardanus cena* butterflies at the UNR site in 2013.

Pollen loads and scale analysis—To quantify pollen loads on visitors to *Clivia* flowers, insects were captured using insect nets and birds captured using mist-nets. Pollen samples from their bodies were taken using blocks of fuchsin gel, which were melted onto glass microscope slides and sealed with a glass coverslip in the field. Pollen grains were later counted using a light microscope at 40× magnification. Insect visitors were all Lepidoptera that had visibly different levels of pollen deposition on the dorsal and ventral surfaces of their wings and bodies, and hence, separate pollen samples were taken from these body parts.

To determine whether contact with butterfly wings is important for pollen deposition in *C. miniata* and *C. gardenii*, we examined stigmas for the co-presence of pollen and butterfly scales. From 40–96 stigmas were collected from each of three sites (UNR for *C. gardenii*, and both UNR and Karkloof for *C. miniata*) and mounted separately on fuchsin gel slides, as described for insect pollen loads.

Single-visit effectiveness—To determine the effectiveness of *P. dardanus cena* as a pollinator of *C. miniata* at the UNR site, where this butterfly was the most common insect visitor, 15 inflorescences were bagged in bud stage (prior to opening) using bridal veil material, and subsequently emasculated prior to anthesis. Once flowers on these plants had opened and their stigmas were receptive, they were exposed to visits by *P. dardanus cena* butterflies. We distinguished between brush visits (n = 4), where pollinators did not alight on the flower but usually made stigmatic contact, and feeding visits (n = 12) where butterflies probed the flowers for nectar. All visits were to a single flower per inflorescence. Immediately after floral contact, the visited flower was removed and its stigma placed on a glass microscope slide in the field, stained with fuchsin gel and sealed with a glass coverslip. Stigmas were examined using a light microscope at 40× magnification to determine the number of pollen grains deposited by floral visitors.

Supplemental hand-pollination and selective pollinator exclusion—At the UNR site, all flowers on 13 plants of *C. miniata* and 7 plants of *C. gardenii* were hand-pollinated (pollen supplementation) as a test for pollen-limitation of seed production. Pollen used for crosses was obtained from plants at least 20 metres away. Eighteen *C. miniata* plants and 27 *C. gardenii* plants were marked to be used as controls. To determine whether small insects contribute to pollination of the study species, 13 *C. miniata* plants and 14 *C. gardenii* plants were covered with plastic mesh cages (apertures: 15 × 12 mm) prior to floral opening. Observations showed that these exclusion cages effectively prevented birds and swallowtail butterflies from gaining access to the flowers. Only small insects such as ants, bees, flies, wasps and small lepidoptera could potentially gain access to the flowers. In a separate experiment to determine if night-flying moths contribute to pollination of *C. miniata*, we bagged inflorescences at the bud stage, and when flowers had opened, exposed all flowers on 6 plants during the day, 11 plants during the night, and later recorded fruit and seed set.

Floral traits—We measured floral traits in the study species to determine which might have been modified during a transition from bird- to insect pollination in *Clivia*. Morphological traits were measured for 84 flowers from 14 plants of *C. miniata*, and 54 flowers from 8 plants of the "robust form" of *C. gardenii*. Perianth length was measured from the tip of the corolla to the base of the ovary. Entrance width was measured at the diameter of the widest point of the flower. The angle of floral curvature was measured, as the number of degrees of deviation from the axis of the pedicel, using a protractor. To calculate the extent of herkogamy, stigma and anther protrusion (at anthesis) from the perianth was measured. Nectar volume and concentration were measured for 56 flowers from 13 plants of *C. miniata* and 108 flowers from 14 plants of the "robust form" of *C. gardenii*. Standing crop of nectar in flowers with receptive stigmas was measured using 50 μl microcapillary tubes. Nectar concentration was measured using a portable Bellingham and Stanley refractometer (0–50%). Nectar sugar composition of *C. miniata* (16 flowers from 16 plants) and *C. gardenii* (11 flowers from 11 plants) was analyzed using a Shimadzu high performance liquid chromatography (HPLC) instrument, as described by Steenhuisen and Johnson (2012). Spectral reflectance of the visible parts of the corolla of *Clivia gardenii*, *C. gardenii* "robust form" and *C. miniata* was measured from 300–700 nm using an Ocean Optics S2000 spectrophotometer (Ocean Optics, Dunedin, Florida, USA) and a fiber optic reflection probe (QR-400-7-UV-VIS; 400 μm) (Johnson and Andersson, 2002).

To determine floral scent composition of the study species, dynamic headspace samples collected from 10 flowering individuals of *C. miniata* (across three sites) and 7 individuals of *C. gardenii* (across two sites). Air from inflorescences enclosed in acetate bags was pumped at 200 mL/min through small filters containing an equal mixture of tenax and activated charcoal. These filters were directly thermally desorbed in a Chromatoprobe device attached to a Varian GC (gas chromatograph) coupled with a Varian 1200 quadrupole mass spectrometer and analyzed according to the protocol described by Shuttleworth and Johnson (2009).

Statistical analyses—All statistical analyses were performed using SPSS Version 19 (IBM Corp.). Duration of butterfly visits and duration of nectar probing by butterflies were analyzed using generalized linear models (GLMs) with a Gamma error distribution and log link functions. Differences in the mean number of pollen grains deposited on stigmas during brush and feeding visits were analyzed using a GLM with a Poisson error distribution and log link function. The significance of GLMs was assessed using likelihood ratios. Regression analyses using log-transformed data were used to explore the relations between the numbers of butterfly wing scales and pollen grains on stigmas. We used generalized estimating equations (GEEs) to analyze the supplemental hand-pollination and selective exclusion experiments to account for lack of independence among flowers on each of the plants used in these experiments. Proportions of flowers setting fruit were analyzed using GEE models with a binomial error distribution model and logit link function. Seed set data were analyzed using GEE models with a negative binomial distribution model and log link function. GEE models used an exchangeable correlation matrix and significance was assessed using Score statistics. To assess the significance of differences among means, we used the Dunn-Šidák method. For graphical presentation of marginal means in the original measurement scale, we back-transformed log or logit data resulting in asymmetrical standard errors. Fecundity data for the day vs. night exclusion experiments were analyzed using Mann-Whitney U tests as these data did not conform to any known distribution. Mean values for floral traits of *C. miniata* and *C. gardenii* were compared using t tests for which equal variances were not assumed. Several flowers were typically measured per plant, but we used the mean values of flower measurements on each plant as replicates. For analysis of the mass of volatiles emitted per plant, we used a GEE with sampling site treated as the subject to account for potential correlated responses among plants growing together, and a Gamma error distribution with an identity link. Wald, rather than Score, statistics were used because of the small sample sizes for this particular analysis.

RESULTS

Pollinator Observations—Flowers of *C. miniata* in the Karkloof and UNR forests were visited by butterflies belonging to Papilionidae and, to a lesser extent, Pieridae and Nymphalidae (Fig. 2A-D; Appendix S1 [see Supplemental Data with the online version of this article], and Appendix S2 [video footage]). *Papilio echerioides echerioides* and *Papilio ophidicephalus* were the most common visitor species at the Karkloof site, while *P. dardanus cena* was the most common visitor at the UNR site (Appendix S1).

Several hundred butterfly visits to *C. miniata* flowers were observed and detailed behavior was recorded for 215 of these floral visitors (Table 1). Overall, butterflies contacted the anthers on 82% of visits and the stigma on 81% of visits. Remarkably, 97% of visits involved the probing of just a single flower

Fig. 2. (A) *Papilio echerioides echerioides* probing a *Clivia miniata* flower in the Karkloof. (B) *Papilio ophidicephalus* shortly after alighting on a *C. miniata* flower in the Karkloof, exhibiting a proboscis extension reflex. (C) *P. dardanus cena* shortly after alighting on a *C. miniata* flower in the UNR, carrying a large load of outcross pollen on the ventral wing surface. (D) *Belenois zochalia zochalia* alighting on a *C. miniata* flower in the UNR. (E) *Cinnyris talatala* (White-belied sunbird) feeding on a *C. gardenii* flower. (F) *Clivia gardenii* "robust form" in the swamp forest of the PC, illustrating two inflorescences on a single individual. Scale bars represent 40 mm.

Table 1. Foraging behavior of various butterfly species visiting *Clivia miniata* flowers in the Karkloof and Umtamvuna Nature Reserve (UNR) sites. Sample size (*n*) indicates observations of different butterfly individuals (see Appendix S1 for a full list of species observed) for which details of their behavior was recorded (nr = not recorded). Means (±SE) for visit duration that share superscript letters within a site are not significantly different (see Results for details).

			Proportion of visits					Duration (s)	
Site	Species	n	brush	alight	anther contact	stigma contact	probed >1 flower/plant	entire visit	nectar probing
Karkloof	*Nepheronia argia*	15	0.00	1.00	1.00	0.87	0.07	9.13 ± 0.15^{ABC}	8.13 ± 0.16^{ABC}
	Papilio nireus lyaeus	3	0.00	1.00	1.00	1.00	0.00	5.67 ± 0.33^{ABC}	4.66 ± 0.36^{ABC}
	P. dardanus cena	4	0.00	1.00	1.00	1.00	0.00	7.75 ± 0.28^{ABC}	6.75 ± 0.31^{ABC}
	P. demodocus demodocus	9	0.00	1.00	1.00	1.00	0.00	6.78 ± 0.19^{ABC}	5.56 ± 0.21^{ABC}
	P.euphranor	10	0.00	1.00	1.00	1.00	0.00	13.20 ± 0.18^{A}	11.60 ± 0.20^{A}
	P. ophidicephalus	31	0.13	0.87	0.94	0.90	0.00	5.45 ± 0.11^{C}	4.58 ± 0.12^{C}
	P. echerioides echerioides	54	0.24	0.76	0.85	0.83	0.04	9.07 ± 0.09^{AB}	8.24 ± 0.10^{AB}
UNR	*Nepheronia argia*	3	nr	nr	0.67	1.00	0.33	2.41 ± 0.44^{A}	0.85 ± 0.57^{A}
	Belenois zochalia zochalia	11	nr	nr	0.73	0.27	0.00	12.31 ± 0.23^{B}	8.78 ± 0.26^{B}
	Papilio dardanus cena	75	0.44	0.56	0.68	0.75	0.03	4.93 ± 0.09^{C}	3.61 ± 0.11^{C}

per plant (Table 1, Appendix S2). There were significant differences among butterfly species in both duration of visits to whole inflorescences (Karkloof: $\chi^2 = 22.679$, $df = 6$, $P < 0.001$; UNR: $\chi^2 = 18.821$, $df = 3$, $P < 0.001$) and single flowers (Karkloof: $\chi^2 = 21.793$, $df = 6$, $P < 0.001$; UNR: $\chi^2 = 16.010$, $df = 2$, $P < 0.001$) (see Table 1 for multiple comparisons).

Nocturnal observations of *C. miniata* flowers revealed very little visitor activity. A few small noctuid moths were observed alighting on *C. miniata* flowers in Karkloof and the UNR, but no probing or feeding bouts were observed.

Observations of populations of the "robust form" of *C. gardenii* in the PC forest throughout the flowering periods of 2007–2012 indicated that *Cyanomitra olivacea* (Olive sunbird, formerly *Nectarinia olivacea*) was the primary pollinator. Approximately 15 visits by sunbirds, which alighted on the stout peduncle of the plants, were observed. Sunbirds began foraging at first light and carried on feeding intermittently throughout the day until dusk. They tended to forage on a single flower on an inflorescence, rather than on multiple flowers on a plant. White-bellied sunbirds (*Cinnyris talatala*, formerly *Nectarinia talatala*) were observed feeding on flowers of the gracile form of *C. gardenii* near Gibba Gorge (Fig. 2E). Claw marks, where sunbirds have gripped the inflorescences while probing flowers for nectar in an upward direction, were often visible on the inflorescence stems of *C. gardenii* at both sites, but were not seen on *C. miniata*. Lepidoptera were rarely seen in swamp forest habitats, and none were seen on flowers of *C. gardenii*.

Pollen loads and scale analysis—Butterflies observed on *C. miniata* flowers carried large pollen loads on their ventral wing surfaces (visible in Fig. 2C), and smaller amounts on their bodies. Owing to the exserted stigma, butterfly visitors deposited pollen from the ventral surface of their wings onto the stigma as they alighted, often just prior to closing their wings (Fig. 2A) and then picked up additional loads of pollen. In addition, contact with stigmas often occurred as papilionid butterflies brushed the flowers during inspections that did not result in alighting (Table 1). Pollen was typically deposited onto the folded wings of the butterflies as they moved deeper into the trumpet-shaped perianth to feed on nectar (Fig. 2A; Appendix S2). Eight individuals of *P. demodocus demodocus* captured at UNR carried a mean (±SE) of 1221.5 ± 438.2 *C. miniata* pollen grains on their ventral wing surface and 107.6 ± 69.1 on their dorsal wing surface, as well as 15.2 ± 6.6 unidentified pollen grains on the ventral wing surface and 10.2 ± 5.1 on the dorsal wing surface. Two *P. echerioides echerioides* butterflies captured in the Karkloof forest carried 210.5 ± 145.5 *C. miniata* pollen grains on their ventral wing surface and 31.5 ± 11.5 on their dorsal wing surface. No pollen of other species was recorded on these individuals. A single male olive sunbird (*Cyanomitra olivacea*) caught in mist nests in the swamp forests carried 127 *C. gardenii* pollen grains on its head and bill (84 and 43 pollen grains, respectively). No other pollen grains were found on this bird. Large deposits of pollen were also visible on the heads and bills of white-bellied sunbirds, including those photographed as they probed flowers of the gracile form of *C. gardenii* (Fig. 2E).

There was a strong positive correlation between the number of lepidopteran wing scales and the number of *C. miniata* pollen grains on *C. miniata* stigmas from the Karkloof and the UNR (Fig. 3). The mean number of *C. miniata* pollen grains per stigma was 76.10 ($n = 51$) for flowers at Karkloof, and the 63.51 ($n = 96$) for flowers at UNR, and there were just 3.02 ± 2.78 and 3.27 ± 1.24 foreign pollen grains per stigma for flowers from these two sites,

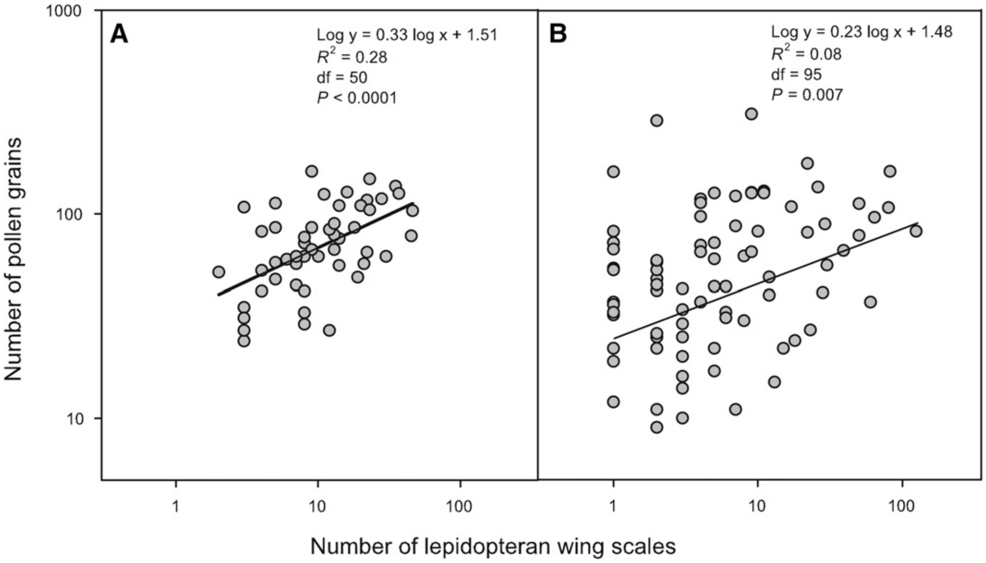

Fig. 3. Relationships between the number of *Clivia miniata* pollen grains and the number of lepidopteran scales found on *C. miniata* stigmas from (A) the Karkloof and (B) the UNR sites. Each stigma was taken from a separate plant.

respectively. No lepidopteran scales were found on stigmas of 40 *C. gardenii* flowers (representing 40 plants). We recorded means of 305.28 ± 24.23 *C. gardenii* pollen grains and 1.43 ± 0.53 foreign pollen grains on stigmas of *C. gardenii* at the UNR site.

Single-visit effectiveness—*Papilio dardanus cena* butterflies effectively deposited pollen on *C. miniata* stigmas during both brush and alighting visits, but the mean number of pollen grains deposited during brush visits (214.6, upper SE 222.1, lower SE 207.4, $n = 4$) was significantly higher than that deposited during alighting visits (56.7, upper SE 58.9, lower SE 54.6, $n = 12$, $\chi^2 = 659.203$, $df = 15$, $P < 0.001$).

Supplemental hand-pollination and selective pollinator exclusion—There were no significant differences in fruit or seed set between open-pollinated controls and pollen-supplemented inflorescences (Fig. 4). For both species, however, plants from which birds and larger butterflies were excluded by means of mesh cages produced significantly lower levels of fruit and seed than did unmanipulated open controls (Fig. 4).

Plants of *C. miniata* exposed to pollinator visits for a single night ($n = 11$) showed significantly lower fecundity than those exposed for a single day ($n = 6$), both in terms of fruits per plant (night: median = 0, day: median = 16.5, $U = 17.50$, $P = 0.041$) and seeds per flower (night: median = 0, day: median = 0.303, $U = 17.00$, $P = 0.036$).

Floral traits—For most morphological traits, there were significant differences in dimensions between the tubular pendulous flowers of *C. gardenii*, and the trumpet-shaped flowers of *C. miniata* (Table 2). Flowers of both species are protogynous and herkogamous (Table 2). Although the mean anther and style lengths in *C. gardenii* are very similar, the style is usually either shorter or longer than the anthers, resulting in a small degree of herkogamy (Table 2). *Clivia gardenii* flowers produce much larger amounts of nectar than do those of *C. miniata* (Table 2). There was, however, no significant difference between the species in terms of the concentrations and sucrose proportions of their nectar (Table 2).

Spectral reflectance was similar for measured flowers of *C. gardenii* "gracile" form, *C. gardenii* "robust form," and *C. miniata* (Fig. 5). The flowers appear orange to humans, but also reflect UV wavelengths (Fig. 5). *Clivia miniata* differs from the two forms of *C. gardenii* in lacking green perianth tips and having distinct cream and yellow "nectar guides" (Fig. 5C).

Clivia miniata emits a simple blend of volatiles, consistently dominated by benzaldehyde, benzyl alcohol, and benzyl benzoate (Table 3), which is readily detectable by the human nose. On average, scent emission from *C. miniat*a inflorescences is about 50-fold greater than it is in *C. gardenii* (Table 3), which is unscented to the human nose. The marginal (adjusted for population effects) mean floral scent emissions recorded were 1547 ± 391 ng/h for *C. miniat*a and 33 ± 0.1 ng/h for *C. gardenii* ($\chi^2 = 14.9$, $df = 1$, $P < 0.001$). None of the other pendulous-flowered *Clivia* species are scented to the human nose and we did not record any volatile emissions in an additional sample of the related bird-pollinated species *C. caulescens*.

DISCUSSION

Evidence for the existence of specialized bird and butterfly pollination systems in *Clivia* presented in this study includes (1) direct observations of floral visitors (Fig. 2, Table 1, Appendix S1, S2); (2) pollen load analysis (see Results); (3) selective-exclusion experiments (Fig. 4); and, (4) in the case of butterfly pollination in *C. miniata*, single-visit experiments and a positive association between butterfly wing-scales and pollen loads on stigmas (Fig. 3). Although available phylogenies of *Clivia* differ with respect to whether *C. gardenii* or *C. caulescens* is placed as sister to *C. miniata*, all of them place *C. miniata* as a product of the most recent bifurcation in the genus (Ran, Hammett, and Murray, 2001; Conrad, Reeves, and

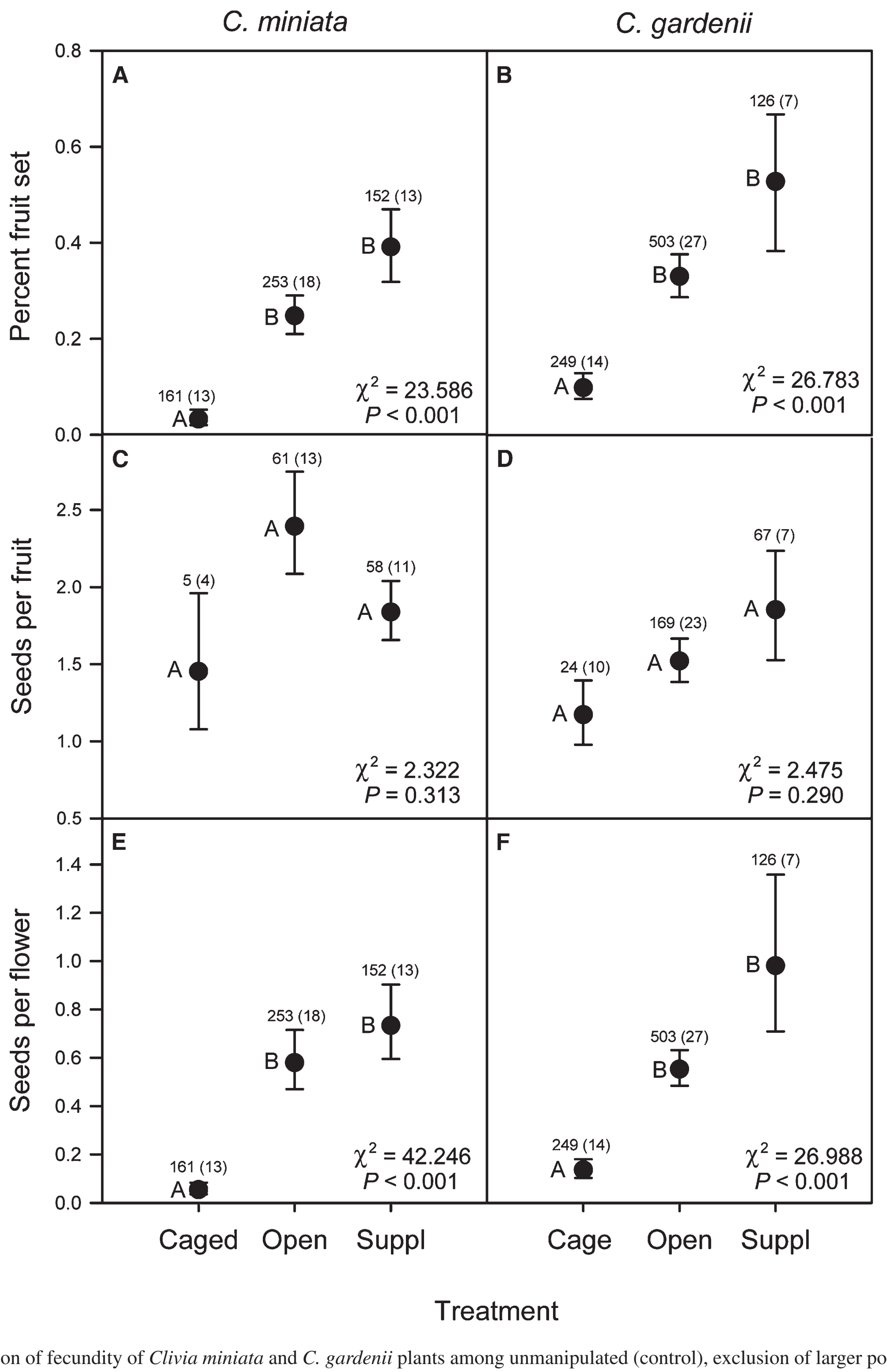

Fig. 4. Comparison of fecundity of *Clivia miniata* and *C. gardenii* plants among unmanipulated (control), exclusion of larger pollinators (caged), and pollen supplementation (Suppl.) treatments. Measures of fecundity for treatment groups are fruit set (A-B) seeds per fruit (C-D) and seeds per flower (E-F). Values above the means (±SE) are the total number of flowers scored and the number of plant replicates (in parentheses). The χ^2 values have two degrees of freedom. Dissimilar capital letters indicate significant differences among treatment means.

Rourke, 2003; Conrad et al., 2006; Conrad, 2008). Although *C. gardenii* was the only species in which we studied bird-pollination in detail, there are numerous published anecdotal observations of bird pollination in all of the *Clivia* species with similar tubular pendulous flowers (Koopowitz, 2002; Rourke, 2002; Manning, 2005). It can thus be inferred that the direction of the pollination system transition in *Clivia* was from bird- to butterfly pollination (Fig. 1). From the characters found in extant

TABLE 2. Comparison of morphological traits and nectar sugar composition of flowers of *Clivia miniata* (UNR site) and *C. gardenii* (PC site). Values are grand means (±SE), with the numbers of measured flowers and plants given in parentheses. The replicates for statistical tests are individual plants.

Trait	*Clivia miniata*	*Clivia gardenii*	*t*	*df*	*P*
Morphology					
Perianth length (mm)	**74.42** ± 1.82 (84, 14)	**39.26** ± 1.16 (54, 8)	16.32	20	< 0.001
Stigma length (mm)	**71.41** ± 1.63 (84, 14)	**40.00** ± 2.10 (54, 8)	11.84	20	< 0.001
Anther length (mm)	**67.39** ± 1.70 (84, 14)	**40.40** ± 1.81 (54, 8)	10.85	20	< 0.001
Herkogamy (mm)	**6.46** ± 0.71 (84, 14)	**3.92** ± 0.95 (54, 8)	2.36	20	0.032
Entrance width (mm)	**73.20** ± 5.28 (84, 14)	**10.43** ± 0.77 (54, 8)	11.77	20	< 0.001
Angle of curvature (deg)	**0.00** ± 0.00 (84, 14)	**9.30** ± 0.64 (54, 8)	14.57	20	< 0.001
Nectar					
Nectar volume (μL)	**4.54** ± 0.74 (56, 13)	**20.55** ± 3.11 (108, 14)	5.77	25	< 0.001
Nectar concentration (%)	**22.27** ± 2.70 (56, 13)	**20.87** ± 1.90 (108, 14)	0.48	25	0.635
Sucrose (% of total sample)	**50.95** ± 2.61 (16, 16)	**61.49** ± 2.56 (11, 11)	1.513	25	0.230

species, we can infer that the major modifications associated with this shift were a change in flower shape and orientation (from tubular pendulous to upright trumpet-shaped), nectar production (a decrease), and scent production (an increase in the amount and number of compounds emitted).

Floral morphology was most obviously modified during the shift from bird- to butterfly pollination in *Clivia.* The upright trumpet-shaped flowers of *C. miniata* provide a landing platform for butterflies and accommodate their wings while they feed on nectar (Fig. 2A-D). There is striking convergence in the upright trumpet-shaped butterfly pollinated flowers that have evolved in various amaryllid lineages in South Africa (Johnson and Bond 1994, and this study). Similar floral morphology also occurs in *Lilium* and *Hemerocallis* species pollinated by butterflies (Edwards and Jordan, 1992; Hirota et al., 2012).

Our observations indicate that the majority of butterflies that visit flowers of *C. miniata* are *Papilio* swallowtails (Appendix S1). *Papilio echerioides echerioides* and *P. dardanus cena*, the two primary visitors of *C. miniata* flowers in the Karkloof and the UNR respectively, made stigma contact with their ventral wing surfaces in more than two thirds of visits to flowers (Table 1). Pollen is deposited on the wings, particularly the ventral surfaces, of butterflies both when they feed on nectar and when they brush over flowers during inspection visits (Appendix S2). Brush visits to flowers by butterflies are associated with mate-searching and territorial behavior, particularly by males. Our results indicate that *Papilio* butterflies that brush over *C. miniata* flowers with their wings deposit more pollen than those that settle to feed on nectar. Butterfly scales were found on virtually every *C. miniata* stigma examined and the numbers of these scales were positively correlated with stigmatic pollen loads (Fig. 3). Pollen deposition on the wings of butterflies has previously been reported for mimusoid legumes (Cruden and Hermann-Parker, 1979), lilies (Barrows, 1979), and various amaryllids (Johnson and Bond, 1994). Analysis of lepidopteran scales on stigmas has been used previously as a proxy for visitation (Rodger, Van Kleunen, and Johnson, 2013), but to our knowledge, this is the first study to correlate the number of lepidopteran wing scales with pollen grains on stigmas and also use single-visit studies to test the hypothesis of pollen transfer by butterfly wings.

The number of pollen grains on stigmas, and even the number deposited in single visits by butterflies, far exceeds the approximately 16 ovules available in *C. miniata* flowers (I. Kiepiel, unpublished data). Despite this, both fruit set and the number of seeds per fruit in this species was low and was not increased by supplemental hand-pollination (Fig. 4). A similar pattern was observed in *C. gardenii* (Fig. 4), suggesting that fecundity in both species is resource-limited rather than pollen-limited.

We found that *Clivia gardenii* was pollinated by sunbirds, a finding which corroborates numerous earlier anecdotal reports of bird pollination in pendulous *Clivia* species (Koopowitz, 2002; Rourke, 2002; Manning, 2005). The orientation and morphology of the flowers of these species appear to discourage butterflies from visiting inflorescences. Lepidoptera that flutter while feeding, such as hawkmoths and swallowtail butterflies, find it very difficult or impossible to feed from pendant flowers as their proboscis can only extend horizontally or downward when the animal hovers. In a study of hawkmoth-pollinated *Aquilegia pubescens*, normally upward-facing flowers that were manipulated into a pendant position received an order of magnitude fewer visits by hawkmoths (Fulton and Hodges, 1999). A selective exclusion experiment for *C. gardenii* revealed that small insects make minimal contributions to seed production (Fig. 2E). This, together with the absence of observations of large insect visitors for this species, strongly supports our finding of bird-pollination in *C. gardenii*. The pendulous tubular flowers of the four bird-pollinated *Clivia* species are strikingly similar to those of other plant species pollinated by sunbirds in South Africa (Geerts and Pauw, 2007). An important feature of all of these sunbird-pollinated plants is the provision of a perch within probing distance of a cluster of flowers (Anderson, Cole, and Barrett, 2005). The floral morphology of *C. miniata*, by comparison, constrains feeding by perching sunbirds by making it virtually impossible for a bird perched on the inflorescence stem to reach around the trumpet shaped flowers to access the nectar.

Plants pollinated by sunbirds generally produce far more nectar than those pollinated by butterflies (Johnson and Bond, 1994) and this difference is also apparent in *Clivia* (Table 2). Unlike nectar volume, nectar concentration and sugar composition do not tend to differ strongly between sunbird and butterfly pollination systems in South Africa (Johnson and Bond, 1994; Johnson and Nicolson, 2008), and this is also reflected in *Clivia* (Table 2). In more general surveys, the concentration of nectar in flowers pollinated by butterflies tends to range from 15–25% (Baker and Baker, 1983; Cruden, Hermann, and Peterson, 1983) and a similar range has been recorded for both hummingbird and sunbird-pollinated flowers (Johnson and Nicolson, 2008). This suggests that bird-pollinated

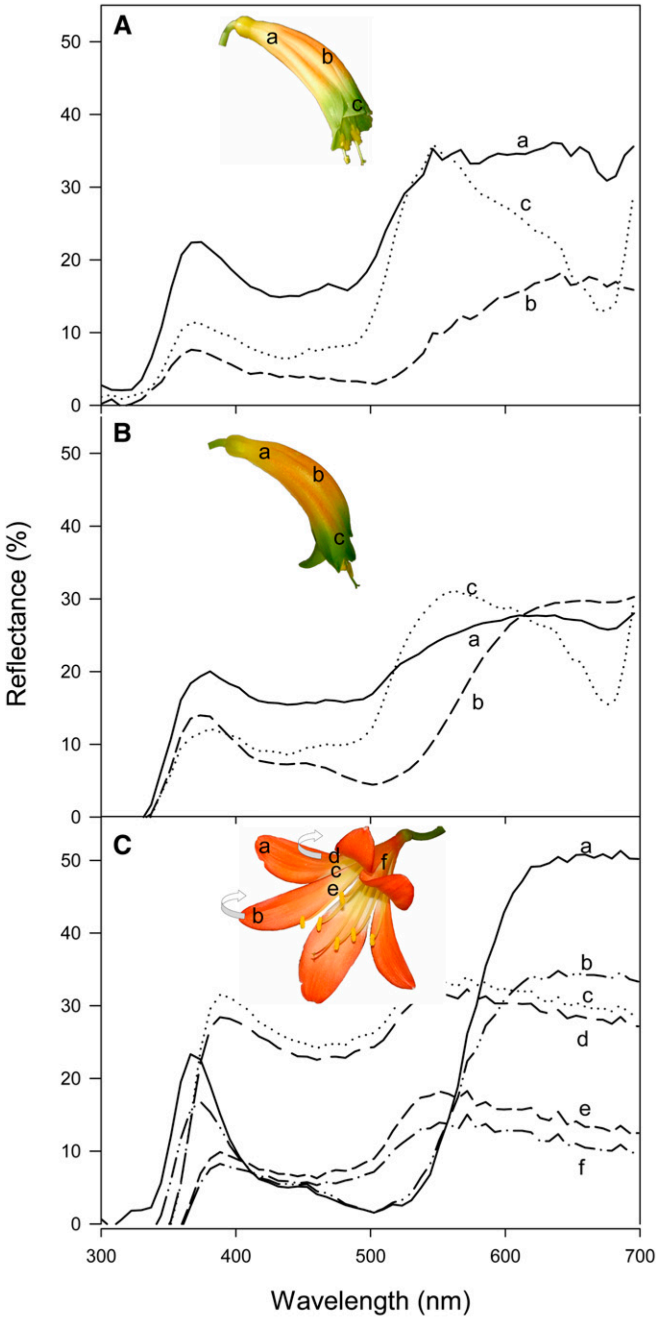

Fig. 5. Spectral reflectance of *Clivia gardenii* (A), *C. gardenii* "robust form" (B) and *C. miniata* (C). Spectra represent mean reflectance for each species (*n* = 6 flowers taken from 6 plants). Pictures and corresponding labels within graphs represent the areas on the corolla from which the measurements were recorded.

flowers would be essentially pre-adapted for a shift to butterfly pollination in terms of nectar concentration. Although both sunbirds (Brown, Downs, and Johnson, 2008) and butterflies (Rusterholz and Erhardt, 1997) have been shown to exhibit some sugar type preferences, they will feed from both hexose and sucrose solutions, and plants pollinated by these two groups vary widely in sugar composition (Brown, Downs, and Johnson, 2008; Johnson and Nicolson, 2008). This suggests that nectar sugar composition in *Clivia* species would not need significant modification during a shift to butterfly pollination.

The parts of the flowers of *C. miniata* and *C. gardenii* that are orange to humans have very similar spectral reflectance patterns (Fig. 5). This spectral reflectance pattern is also found in other bird-pollinated *Clivia* species (I. Kiepiel, unpublished data), suggesting that overall flower color was little modified during the shift from bird- to butterfly pollination. The main modification was the evolution of a yellow throat at the base of the inner tepal of *C. miniata*. This yellow patch presumably acts as a nectar guide, and is strikingly similar to that in other butterfly pollinated petaloid monocots, such as *Lilium philadelphicum* (Barrows, 1979). Both sunbirds and butterflies rely heavily, and in the case of sunbirds, probably exclusively, on visual cues for locating flowers (Lewis and Lipani, 1990; Johnson and Bond, 1994; Omura and Honda, 2005). Many butterflies show innate preferences toward certain floral colors (Ilse and Vaidya, 1956; Swihart and Swihart, 1970) but are also capable of rapid associative learning, linking food rewards with colors (Weiss, 1997). The strong UV reflectance peak of *Clivia* flowers (Fig. 5) is unusual among flowers that are orange to humans and likely influences the color as perceived by both sunbirds and butterflies, as UV receptors are found in both animal groups (Eguchi et al., 1982; Endler and Mielke, 2005).

Flowers of the two bird-pollinated *Clivia* species examined here are effectively unscented (Table 3). Lack of scent or weak scent emission from flowers of bird-pollinated flowers was documented in several studies (Knudsen et al., 2004; Steenhuisen, Raguso, and Johnson, 2012). This is consistent with the established idea that olfactory perception in most birds is poor (Bang and Cobb, 1968) although it is known that some birds use scent in navigation (Wiltschko, 1996) and foraging for food (Nevitt, Veit, and Kareiva, 1995). Flowers of the butterfly pollinated *C. miniata* emit a very simple blend of volatiles (Table 3). Some butterflies use scent together with color cues to find flowers (Andersson, 2003) and scent can also play a role in flower constancy in butterflies (Goulson and Cory, 1993; Weiss, 1997; Andersson, 2003). In *Brassica rapa*, a combination of color and scent was found to stimulate floral visits by the white cabbage butterfly, *Pieris rapae* (Ômura, Honda, and Hayashi, 1999). Benzaldehyde and benzyl alcohol, which are emitted by *C. miniata*, are known to be detected by certain butterflies (Topazzini, Mazza, and Pelosi, 1990; Omura, Honda, and Hayashi, 1999). Nevertheless, Andersson et al. (2002) did not find any clear convergence in volatile profiles among butterfly pollinated plants. The relatively small number of compounds in the blend emitted by flowers of *C. miniata* may reflect lack of scent production by the immediate ancestors of this species.

In conclusion, this study identifies a shift from bird- to butterfly pollination in *Clivia* and highlights floral morphology, nectar volume, and floral scent emission as traits that were likely to have been modified by selection during this transition. Flower color, nectar volume, and nectar sugar composition, by contrast, seem, to have been little modified and may even have served as pre-adaptations that facilitated the transition. It is likely that bird- to butterfly pollination system transitions are underreported and further examples are thus likely to emerge, particularly from studies of tropical floras.

Table 3. Floral volatile emissions of entire intact inflorescences of *Clivia miniata* and *C. gardenii* sampled in various locations in KwaZulu-Natal, South Africa (see text for statistical analysis).

	C. miniata				*C. gardenii*	
Compound	Karkloof (n = 3)†	Karkloof (n = 3)‡	Garden (n = 3)‡	UKZN (n = 1) *‡	PC (n = 1)‡	GG (n = 6)‡
Limonene	0.98 (3)	0.08 (1)	1.68 (2)	—	—	—
1-octen-3-ol	—	—	—	—	—	100 (6)
Benzaldehyde	11.61 (3)	36.49 (3)	38.43 (3)	55.39 (1)	—	—
Linalool	14.94 (2)	0.08 (2)	—	0.69 (1)	—	—
Benzyl acetate	—	—	0.23 (1)	—	—	—
Benzyl alcohol	70.24 (3)	58.82 (3)	59.65 (3)	30.40 (1)	65.59 (1)	—
Phenylethyl alcohol	—	—	—	—	34.41 (1)	—
Benzyl benzoate	1.87 (3)	4.53 (3)	0.01 (1)	13.52 (1)	—	—
Benzyl 3-methylbutanoate	0.36 (3)	—	—	—	—	—
Emission rate (ng/h)	744.8	2396.1	1903.2	121.7	33.1	33.9

Notes: * = *C. miniata* var. *citrina*; † = DB5 column; ‡ = DB-Wax column. UKZN = University of KwaZulu-Natal, PC = Pondoland Centre of Endemism; GG = Gibba gorge. Garden = cultivated plants in Pietermaritzburg.

LITERATURE CITED

Aigner, P. A. 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? *Oikos* 95: 177–184.

Anderson, B., W. W. Cole, and S. C. H. Barrett. 2005. Specialized bird perch aids cross-pollination. *Nature* 435: 41–42.

Andersson, S. 2003. Foraging responses in the butterflies *Inachis io, Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae) to floral scents. *Chemoecology* 13: 1–11.

Andersson, S., L. A. Nilsson, I. Groth, and G. Bergstrom. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Botanical Journal of the Linnean Society* 140: 129–153.

Baker, H. G., and I. Baker. 1983. Floral nectar sugar constituents in relation to pollinator type. *In* C. E. Jones and R. J. Little [eds.], Handbook of experimental pollination biology, 117-141. Van Nostrand Reinhold Co., New York, USA.

Bang, B. G., and S. Cobb. 1968. The size of the olfactory bulb in 108 species of birds. *The Auk* 85: 55–61.

Barrows, E. M. 1979. Flower biology and arthropod associate of *Lilium philadelphicum. Michigan Botanist* 18: 109–115.

Brown, M., C. T. Downs, and S. D. Johnson. 2008. Sugar preferences of nectar feeding birds—a comparison of experimental techniques. *Journal of Avian Biology* 39: 479–483.

Conrad, F. 2008. Molecular systematics, biogeography and dating of the tribe Haemantheae (Amaryllidaceae) and the phylogeography of *Clivia*, University of Cape Town, Cape Town, South Africa.

Conrad, F., G. Reeves, and J. P. Rourke. 2003. Phylogenetic relationships of the recently discovered species—*Clivia mirabilis. South African Journal of Botany* 69: 204–206.

Conrad, F., G. Reeves, D. A. Snijman, and T. A. J. Hedderson. 2006. Genetic relationships within the tribe Haemantheae (Amaryllidaceae) based on plastd DNA sequence data. *In* S. A. Ghazanfar and H. J. Beentje [eds.], Taxonomy and ecology of African plants, their conservation and sustainable use, 73–83. Royal Botanic Gardens Kew, London, UK.

Cruden, R. W., and S. M. Hermann-Parker. 1979. Butterfly pollination of *Caesalpinia pulcherrima*, with observations on a psychophilous syndrome. *Journal of Ecology* 67: 155–168.

Cruden, R. W., S. M. Hermann, and S. Peterson. 1983. Patterns of nectar production and plant pollinator coevolution. *In* B. Bentley and T. S. Elias [eds.], The biology of nectaries, 80–125. Columbia University Press, New York, USA.

Edwards, J., and J. R. Jordan. 1992. Reversible anther opening in *Lilium philadelphicum* (Liliaceae): a possible means of enhancing male fitness. *American Journal of Botany* 79: 144–148.

Eguchi, E., K. Watanabe, T. Hariyama, and K. Yamammoto. 1982. A comparison of electrophysiologically determined spectral responses in 35 species of Lepidoptera. *Journal of Insect Physiology* 28: 675–682.

Endler, J. A., and P. W. Mielke. 2005. Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society. Linnean Society of London* 86: 405–431.

Faegri, K., and L. van der Pijl. 1979. The Principles of pollination ecology. Pergamon, Oxford, UK.

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

Fulton, M., and S. A. Hodges. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 266: 2247–2252.

Geerts, S., and A. Pauw. 2007. A malachite sunbird pollination guild in the Cape flora, with implications for the endangered *Brunsvigia litoralis. South African Journal of Botany* 73: 289.

Goulson, D., and J. S. Cory. 1993. Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi. Ecological Entomology* 18: 315–320.

Gübitz, T., M. E. Hoballah, A. Dell'Olivo, and C. Kuhlemeier. 2009. *Petunia* as a model system for the genetics and evolution of pollination syndromes. *In* T. Gerats and J. Strommer [eds.], Petunia, 29–49. Springer, New York, USA.

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

Hirota, S. K., K. Nitta, Y. Kim, A. Kato, N. Kawakubo, A. A. Yasumoto, and T. Yahara. 2012. Relative role of flower color and scent on pollinator attraction: experimental tests using F1 and F2 hybrids of daylily and nightlily. *PLoS ONE* 7: e39010.

Ilse, D., and V. G. Vaidya. 1956. Spontaneous feeding response to colours in *Papilio demoleus* L. *Proceedings of the Indian Academy of Science, Section B. Plant Science* 43: 23–31.

Johnson, S. D., and W. J. Bond. 1994. Red flowers and butterfly pollination in the fynbos of South Africa. *In* M. Arianoutsou and R. H. Groves [eds.], Plant-animal interactions in Mediterranean-type ecosystems, 137–148. Kluwer Academic Publishers, Dordrecht, Netherlands.

Johnson, S. D., and S. Andersson. 2002. A simple field method for manipulating ultraviolet reflectance of flowers. *Canadian Journal of Botany-Revue Canadienne De Botanique* 80: 1325–1328.

Johnson, S. D., and S. W. Nicolson. 2008. Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biology Letters* 4: 49–52.

Knudsen, J. T., L. Tollsten, I. Groth, G. Bergstrom, and R. A. Raguso. 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society* 146: 191–199.

Koopowitz, H. 2002. *Clivias*. Timber Press, Portland, USA.

Lewis, A. C., and G. A. Lipani. 1990. Learning and flower use in butterflies: hypotheses from honey bees. *In* E. A. Bernays [ed.], Insect-plant interactions, 95–110. CRC Press, Boca Raton, Florida, USA.

MANNING, J. 2005. *Pollination in Clivia Clivia* 7: 17–22.

MANNING, J. C., AND P. GOLDBLATT. 2005. Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. *International Journal of Plant Sciences* 166: 459–474.

MEEROW, A. W., M. F. FAY, C. L. GUY, Q.-B. LI, F. Q. ZAMAN, AND M. W. CHASE. 1999. Systematics of Amaryllidaceae based on cladistic analysis of plastid sequence data. *American Journal of Botany* 86: 1325–1345.

MURRAY, B. G., Y. RAN, P. J. DE LANGE, K. R. W. HAMMETT, J. T. TRUTER, AND Z. H. SWANEVELDER. 2004. A new species of *Clivia* (Amaryllidaceae) endemic to the Pondoland Centre of Endemism, South Africa. *Botanical Journal of the Linnean Society* 146: 369–374.

NEVITT, G. A., R. R. VEIT, AND P. KAREIVA. 1995. Dimethyl sulphide as a foraging cue for Antarctic procellariiform seabirds. *Nature* 376: 680–682.

OMURA, H., AND K. HONDA. 2005. Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* 142: 588–596.

OMURA, H., K. HONDA, AND N. HAYASHI. 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. *Journal of Chemical Ecology* 25: 1895–1906.

PERRET, M., A. CHAUTEMS, R. SPICHIGER, T. G. BARRACLOUGH, AND V. SAVOLAINEN. 2007. The geographical pattern of speciation and floral diversification in the neotropics: The tribe Sinningieae (Gesneriaceae) as a case study. *Evolution; International Journal of Organic Evolution* 61: 1641–1660.

RAIMONDO, D., L. VON STADEN, W. FODEN, J. E. VICTOR, N. A. HELME, R. C. TURNER, D. A. KAMUNDI, AND P. A. MANYAMA. 2009. Red List of South African Plants. South African National Biodiversity Institute, Pretoria, South Africa.

RAN, Y., K. R. W. HAMMETT, AND B. G. MURRAY. 2001. Phylogenetic analysis and karyotype evolution in the genus *Clivia* (Amaryllidaceae). *Annals of Botany* 87: 823–830.

RODGER, J., M. VAN KLEUNEN, AND S. D. JOHNSON. 2013. Pollinators, mates and Allee effects: the importance of self-pollination for fecundity in an invasive lily. *Functional Ecology* 27: 1023–1033.

ROURKE, J. P. 2002. *Clivia mirabilis* (Amaryllidaceae: Haemantheae) a new species from Northern Cape, South Africa. *Bothalia* 32: 1–7.

ROURKE, J. P.. 2012. South African National Biodiversity Institute, Personal communication.

RUSTERHOLZ, H.-P., AND A. ERHARDT. 1997. Preferences for nectar sugars in the peacock butterfly, *Inachis io. Ecological Entomology* 22: 220–224.

SCHEMSKE, D. W., AND H. D. BRADSHAW JR. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus). Proceedings of the National Academy of Sciences, USA* 96: 11910–11915.

SHUTTLEWORTH, A., AND S. D. JOHNSON. 2009. A key role for floral scent in a wasp-pollination system in *Eucomis* (Hyacinthaceae). *Annals of Botany* 103: 715–725.

SHUTTLEWORTH, A., AND S. D. JOHNSON. 2010. The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems. *Proceedings. Biological Sciences* 277: 2811–2819.

STEBBINS, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307–326.

STEENHUISEN, S.-L., R. A. RAGUSO, AND S. D. JOHNSON. 2012. Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): chemistry. emission rates and function. *Phytochemistry* 84: 78–87.

STEENHUISEN, S., AND S. D. JOHNSON. 2012. Evidence for beetle pollination in the African grassland sugarbushes (*Protea*: Proteaceae). *Plant Systematics and Evolution* 298 857–869.

SWANEVELDER, D., AND R. C. FISHER. 2009. *Clivia*: nature and nurture. Briza Publications, Pretoria, South Africa.

SWANEVELDER, Z. H. 2003. Diversity and population structure of *Clivia miniata* Lindl. (Amaryllidaceae): evidence from molecular genetics and ecology. M.Sc. dissertation, University of the Free State, Bloemfontein, South Africa.

SWIHART, C. A., AND S. L. SWIHART. 1970. Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius* Linn. *Animal Behaviour* 18: 60–64.

TOPAZZINI, A., M. MAZZA, AND P. PELOSI. 1990. Electroantennogram responses of five Lepidoptera species to 26 general odourants. *Journal of Insect Physiology* 36: 619–624.

TRIPP, E. A., AND P. S. MANOS. 2008. Is floral specialization and evolutionary dead end? pollination system transitions in *Ruellia* (Acanthaceae). *Evolution; International Journal of Organic Evolution* 62: 1712–1737.

VALENTE, L. M., J. C. MANNING, P. GOLDBLATT, AND P. VARGAS. 2012. Did pollination shifts drive diversification in southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *American Naturalist* 180: 83–98.

VAN DER NIET, T., AND S. D. JOHNSON. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution* 27: 353–361.

WEISS, M. R. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Animal Behaviour* 53: 1043–1053.

WHITTALL, J. B., AND S. A. HODGES. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447: 706–709.

WILTSCHKO, R. 1996. The function of olfactory input in pigeon orientation: does it improve navigational information or play another role? *The Journal of Experimental Biology* 199: 113–119.

APPENDIX 1

Kiepiel and Johnson —*American Journal of Botany* 101(1): 190-200. 2014. – Data Supplement S1

Appendix S1. Lepidoptera observed feeding on *Clivia miniata* flowers in the Karkloof and Umtamvuna Nature Reserve (UNR) forests.

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Family	Subfamily	Species	Common name	Number of observations	
				Karkloof	UNR
Nymphalidae	Biblidinae	*Eurytela hiarbas angustata*	Pied Piper	0	2
	Danainae	*Amauris ochlea ochlea*	Novice	0	1
	Heliconiinae	*Acraea boopis boopis*	Rainforest Acraea	0	2
		Hyalites cerasa cerasa	Tree-top Acraea	0	2
	Limenitinae	*Cymothoe coranus coranus*	Blond Glider	0	1
		Pseudacraea boisduvalii trimenii	Boisduval's False Acraea	0	1
	Nymphalinae	*Junonia natalica natalica*	Brown Pansy	0	1
Papilionidae	Papilionidae	*Papilio demodocus demodocus*	Citrus Swallowtail	9	2
		Papilio dardanus cena	Mocker Swallowtail	4	97
		Papilio echerioides echerioides	White-banded swallowtail	54	0
		Papilio euphranor	Forest Swallowtail	10	1
		Papilio nireus lyaeus	Green-banded Swallowtail	3	4
		Papilio ophidicephalus	Emperor Swallowtail	31	4
Pieridae	Pierinae	*Eronia leda*	Autumn-leaf Vagrant	0	2
		Nepheronia argia	Large Vagrant	15	5
		Colotis erone	Coastal Purple Tip	0	3
		Belenois thysa thysa	False Dotted Border	0	1
		Belenois zochalia zochalia	Forest White	0	12
		Belenois aurota aurota	Brown-veined White	0	1
		Belenois gidica abyssinica	African veined White	0	1

CHAPTER 3

BREEDING SYSTEMS IN *CLIVIA* (AMARYLLIDACEAE): LATE-ACTING SELF-INCOMPATIBILITY AND ITS FUNCTIONAL CONSEQUENCES

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Breeding systems in *Clivia* (Amaryllidaceae): late-acting self-incompatibility and its functional consequences

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Late-acting (ovarian) self-incompatibility, characterized by minimal or zero seed production following self-pollen tube growth to the ovules, is expected to show phylogenetic clustering, but can otherwise be difficult to distinguish from early-acting inbreeding depression. In Amaryllidaceae, late-acting self-incompatibility has been proposed for *Narcissus* (Narcisseae) and *Cyrtanthus* (Cyrtantheae). Here, we investigate whether it occurs in the horticulturally important genus *Clivia* (Haemantheae) and test whether species in this genus experience ovule discounting in wild populations. Seed-set results following controlled hand pollinations revealed that *Clivia miniata* and *C. gardenii* are largely self-sterile. Self- and cross-pollinated flowers of both species had similar proportions of pollen tubes entering the ovary, and those of *C. gardenii* also did not differ in the proportions of pollen tubes that penetrated ovules, thus ruling out classical gametophytic self-incompatibility acting in the style, but not early inbreeding depression. Flowers that received equal mixtures of self- and cross-pollen set fewer seeds than those that received cross-pollen only, but it was unclear whether this effect was a result of ovule discounting or interactions on the stigma. The prevention of self-pollination by the emasculation of either single flowers or whole inflorescences in wild populations did not affect seed set, suggesting that ovule discounting is not a major natural limitation on seed production. Flowers typically produce one to three large fleshy seeds from approximately 16 available ovules, even when supplementally hand pollinated, suggesting that fecundity is mostly resource limited. The results of this study suggest that *Clivia* spp. are largely self-sterile as a result of either a late-acting self-incompatibility system or severe early inbreeding depression, but ovule discounting caused by self-pollination is not a major constraint on fecundity. © 2014 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2014, **175**, 155–168.

ADDITIONAL KEYWORDS: *Clivia gardenii* – *Clivia miniata* – late-acting self-sterility – ovule discounting – pollen limitation – resource limitation.

INTRODUCTION

Most angiosperms have hermaphrodite flowers and *c*. 50% of these have various mechanisms of genetic self-incompatibility (SI) that prevent self-fertilization (Darlington & Mather, 1949; Brewbaker, 1959; de Nettancourt, 1977). It is now clear that SI evolved numerous times during angiosperm evolution (de Nettancourt, 1997). Resolving the site and timing of self-rejection is important for understanding the origin of SI in plants (Sage *et al*., 1999), understanding the functional consequences of SI (Vaughton & Ramsey, 2010) and for overcoming SI in breeding programmes (Hinata, Isogai & Isuzugawa, 1994). Particularly controversial is late-acting self-incompatibility (LSI), in which rejection is proposed to occur in the ovary rather than the stigma or style (Sears, 1937; Knight & Rogers, 1955; Cope, 1962; Dulberger, 1964; Seavey & Bawa, 1986; Sage, Bertin & Williams, 1994; Sage *et al*., 1999). Recent research has indicated that both pre-zygotic (Kenrick, Kaul & Williams, 1986; Beardsell, Knox & Williams, 1993; Sage *et al*., 1999; Sage, Price & Waser, 2006; Chen *et al*., 2012; Hao *et al*., 2012) and post-zygotic (Sage & Williams, 1991; Gibbs & Bianchi, 1993; Gibbs, Oliveira & Bianchi, 1999; Bittencourt, Gibbs & Semir, 2003; Sage & Sampson, 2003; Bittencourt & Semir, 2005) mechanisms may be involved in LSI. Although the genetics of LSI are not well understood, some progress has been made in work on *Asclepias exaltata*

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L. (Apocynaceae) (Lipow & Wyatt, 2000) and *Ipomopsis tenuifolia* (Gray) V. Grant (Polemoniaceae) (LaDoux & Friar, 2006). Nevertheless, it is notoriously difficult to unambiguously distinguish LSI from early-acting inbreeding depression. In general, LSI manifests as uniform zygote failure before cell division, whereas inbreeding depression manifests as embryo failure at virtually any developmental stage (Charlesworth, 1985; Seavey & Bawa, 1986; Hao *et al*., 2012). Continuing research into LSI is slowly establishing its legitimacy as a distinct form of SI (Seavey & Bawa, 1986; Sage *et al*., 1999; Vaughton & Ramsey, 2010; Vaughton, Ramsey & Johnson, 2010; Chen *et al*., 2012; Ford & Wilkinson, 2012). Studies of self-sterility in *Ipomopsis aggregata* (Pursh) V.Grant (Polemoniaceae) and *Narcissus triandrus* L. (Amaryllidaceae) indicate that ovules of self-pollinated flowers may even degenerate prior to penetration by self-pollen tubes, suggesting that some form of signalling may be involved (Sage *et al*., 1999, 2006). More commonly, however, ovules fail to develop or develop only until pistils abscise, following penetration by self-pollen tubes (Dulberger, 1964; Gibbs & Bianchi, 1999; Bittencourt *et al*., 2003; Sage & Sampson, 2003; Vaughton *et al*., 2010). A recent study on *Theobroma cacao* L. (Malvaceae), undertaken using live-cell confocal microscopy (Ford & Wilkinson, 2012), has confirmed the finding of Cope (1962) that this species contains an idiosyncratic form of LSI, whereby the fusion of incompatible gametes is prevented in the embryo sac.

Believed to have evolved multiple times in angiosperm evolutionary history and in numerous taxa (Seavey & Bawa, 1986; Allen & Hiscock, 2008), LSI is unique among SI systems in that self-pollination can impose a heavy cost in terms of loss of ovules, referred to variously as 'ovule usurpation' (Waser & Price, 1991) and 'ovule discounting' (Barrett, Lloyd & Arroyo, 1996). This has been demonstrated by experiments showing that seed set in plants with LSI is drastically reduced when self-pollen is applied to stigmas before or at the same time as cross-pollen (Cope, 1962; Dulberger, 1964; Waser & Price, 1991; Lloyd & Wells, 1992; Broyles & Wyatt, 1993; Barrett *et al*., 1996; Sage *et al*., 1999; Vaughton & Ramsey, 2010; Vaughton *et al*., 2010). Ovule discounting is, however, not unique to LSI systems and can also occur in plants with strong early-acting inbreeding depression (Lloyd, 1992; Herlihy & Eckert, 2002).

One of the strongest lines of evidence for LSI is that it appears to be clustered in certain plant lineages, including, notably, Apocynaceae, Bignoniaceae, Malvaceae, Fabaceae and Xanthorrhoeaceae (Seavey & Bawa, 1986; Naaborgh & Willemse, 1991; Broyles & Wyatt, 1993; Gibbs & Bianchi, 1999), rather than being widely dispersed phylogenetically, as would be expected if the phenomenon was caused by inbreeding depression. Members of Amaryllidaceae were considered in some early studies to possess gametophytic SI (Pandey, 1960), but LSI has since been reported in *Narcissus tazetta* L. (Dulberger, 1964), *N. triandrus* (Bateman, 1954; Sage *et al*., 1999) and *Cyrtanthus breviflorus* Harv. (Vaughton *et al*., 2010). Breeding systems of Amaryllidaceae are generally poorly documented, particularly in the Southern Hemisphere, where the majority of species are located. Anecdotal records for the horticulturally important southern African amaryllid genus *Clivia* Lindl. (Amaryllidaceae) suggest that some of the species are partially self-compatible (Duncan, 1999; Koopowitz, 2002; Manning, 2005), with up to three seeds per berry being produced when plants of *C. mirabilis* Rourke are self-pollinated (Swanevelder & Fisher, 2009). On the whole, however, horticulturalists have reported that self-pollination in *Clivia* results in a substantial reduction in, and even absence of, seed set when compared with cross-pollination (Duncan, 1999; van der Merwe, Robbertse & de Kock, 2005). Given that the compatibility systems in *Clivia* spp. may also have been subject to selection by breeders, we opted to study the breeding systems of two *Clivia* spp., *C. miniata* (Lindl.) Bosse and *C. gardenii* Hook., using natural populations wherever possible. The aims of this research were: (1) to determine the level of self-fertility and potential for autonomous self-pollination in *Clivia*; (2) to test whether self-pollen tubes enter the ovary and penetrate the ovules; and (3) to determine whether self-pollination results in ovule discounting with an impact on seed production.

MATERIAL AND METHODS

STUDY SPECIES AND SITES

Clivia is a small genus of evergreen perennials in the African tribe Haemantheae (Meerow *et al*., 1999; Rourke, 2002) endemic to southern Africa (Murray *et al*., 2004). *Clivia miniata* is found in the eastern part of southern Africa where plants occur naturally on sandstone and doleritic humic scree in a wide variety of conditions ranging from coastal to subtropical forest habitats (Duncan, 1999; Winter, 2000; van der Merwe *et al*., 2005). Flowering occurs in the Austral spring between August and November (Duncan, 1999; Swanevelder, 2003; van der Merwe *et al*., 2005). *Clivia miniata* is the only member of the genus with upright trumpet-shaped flowers (Swanevelder, 2003). Inflorescences typically comprise between ten and 40 flowers on large umbels (Swanevelder, 2003). Flowers are herkogamous with a style that protrudes beyond the anthers. *Clivia*, *Scadoxus* Raf., *Haemanthus* L. and *Cryptostephanus*

Welw. ex Baker possess a plesiomorphic condition in tribe Haemantheae in which there are fewer than ten ovules per locule (Conrad, 2008). Conversely, the derived state of more than ten ovules per locule, found in *Apodolirion* Baker and *Gethyllis* L., reflects a synapomorphic condition in the tribe (Conrad, 2008). *Clivia miniata* typically produces one to four seeds per fruit which take 9–12 months to mature (Swanevelder, 2003).

Clivia gardenii occurs in coastal and scarp-forest habitats in the eastern part of southern Africa. We studied both the typical form and unusually robust plants, growing in swamp habitats, that some botanists have recognized as C*livia robusta* B.G.Murray, Ran, de Lange, Hammett, Truter & Swanev. (Murray *et al*., 2004). We refer to these latter plants as the 'robust' variety of *C. gardenii,* as many botanists working on *Clivia* do not consider the available evidence sufficient for them to be recognized as a distinct species (Conrad, 2008; J. Rourke, South African National Biodiversity Institute, personal communication). Robust *C. gardenii* plants are distributed in fragmented populations endemic to a small area on the east coast of South Africa, known as the Pondoland Centre (PC) of endemism (Van Wyk, 1994; Van Wyk & Smith, 2001). Flowering is in the Austral autumn from late March to early August or autumn to winter (Swanevelder, 2003; Murray *et al*., 2004). Plants of the robust form of *C. gardenii* typically have 15–40 pendulous tubular flowers per umbel (Swanevelder, 2003; Murray *et al*., 2004). One of the key differences cited by Murray *et al*. (2004) to distinguish the two 'species' is that stigma and anther protrusion from the flower in the gracile form of *C. gardenii* was found to be pronounced, whereas, in the robust variety of *C. gardenii*, stigmas scarcely protrude from the corolla and the anthers are retained within the flower. Floral tube length varies from 30 to 55 mm (Swanevelder, 2003; Murray *et al*., 2004) and flowers are herkogamous. Flowers of *C. gardenii* have less than ten ovules per locule (Conrad, 2008), typically producing one or two seeds per fruit which take 9–12 months to mature (Murray *et al*., 2004).

This study was conducted from 2007 to 2012 at three different field sites in KwaZulu-Natal. Two *C. miniata* populations were studied: one in the Umtamvuna Nature Reserve (UNR) and one in the Karkloof region. A population of the robust form of *C. gardenii* was studied on a private farm north of the UNR. In addition, cultivated plants of *C. miniata* and the non-robust form of *C. gardenii* were used in some of the experiments. As *Clivia* is a protected genus and populations are threatened in the wild, detailed GPS co-ordinates of the study populations are omitted.

Pollen tube analysis

To adequately determine the site and timing of self-sterility, detailed studies of pollen tube growth in cross- and self-pollinated pistils were undertaken using fluorescence microscopy (Martin, 1959; Cruzan, 1986; Sage *et al*., 1999; Bittencourt *et al*., 2003; Vaughton *et al*., 2010). Following the method used by Cruzan (1986), the numbers of germinated pollen grains on the stigma and the number of pollen tubes present at various landmarks along the pistil, including the ovary and ovules, were recorded. Flowers of *C. miniata* and *C. gardenii* (robust form) were emasculated prior to anthesis, bagged in fine net bags and allowed to open naturally. After hand pollination, inflorescences were again bagged, and both self- and cross-pollinated pistils were harvested at various times (i.e. 1 h, 2 h, 5 h, 24 h, 48 h). Additional cross- and self-pollinated pistils were harvested after 72 h for *C. miniata* and *C. gardenii*, respectively. Pistils were harvested in the field and the biological activity of pollen tubes was halted (fixed) by placing them in Carnoy's solution (ratio of six parts 95% ethanol to three parts chloroform to one part glacial acetic acid) for 24 h, followed by immersion in 70% alcohol solution. Pistils were rehydrated in 30% ethanol for 10 min, followed by two rinses of distilled water for 10 min each, followed by a soak for 1 h. Pistils were then bleached in a 0.8 M NaOH for 2 h at 60 °C. Pistils were again rinsed three times for 20 min each in changes of distilled water to remove NaOH. Prior to staining, as much as possible of the superfluous ovary tissue was removed. Pistils were stained using a 0.1% aniline blue solution in 0.1 M K_2HPO_4 for 12 h, mounted in a drop of stain on a glass slide and covered in a drop of glycerol, and then squashed under a coverslip.

Breeding system

The dependence of *C. miniata* and *C. gardenii* (robust form) on pollinators for seed set and their degree of self-sterility were determined using the following controlled hand pollination experiments conducted at the Umtamvuna sites. Prior to flower opening, inflorescences on ten plants of *C. miniata* and nine plants of *C. gardenii* were covered in fine net bags supported by wire. On opening, each of the flowers on an inflorescence was allocated to one of three treatments: (1) unmanipulated as a control for autogamy; (2) self-pollinated; or (3) cross-pollinated using pollen taken from plants at least 20 m distant. Hand pollination was carried out at least twice on each flower, prior to anther dehiscence, when the stigma-lobes were open. *Clivia miniata* and *C. gardenii* seeds were collected when mature, after 9 and 11 months, respectively.

POTENTIAL FOR OVULE DISCOUNTING

Pollen mixture studies

The potential for ovule discounting following self-pollination was investigated in *C. miniata* at the Karkloof site and in *C. gardenii* (non-robust form) at the Pietermaritzburg site by comparing seed set among flowers that received various mixtures of self- and cross-pollen and those that were naturally pollinated. For controlled hand pollinations, inflorescences on 39 plants of *C. miniata* and nine plants of *C. gardenii* were covered in fine net bags supported by wire prior to opening. On opening, each of the flowers on an inflorescence was randomly allocated to receive: (1) cross-pollen; (2) an equal mixture of cross- and self-pollen; (3) an equal mixture of cross- and dead pollen; and (4) self-pollen. For cross-pollination, undehisced anthers from each of two plants, taken at least 20 m distant, were placed in a 1.5-mL Eppendorf tube (Vaughton *et al*., 2010). After dehiscence, pollen was mixed thoroughly before saturating stigmas using toothpicks. For the mixed cross- and self-pollination treatments, two anthers with cross-pollen and two with self-pollen were combined in an Eppendorf tube. For the mixed cross- and dead self-pollen, two anthers with dead pollen were combined with two anthers with cross-pollen. Dead pollen was obtained by microwaving anthers at 800 W for 2 min (Vaughton *et al*., 2010). Dead pollen was applied in order to act as a control for mechanical effects of self-pollen on the success of cross-pollen in the cross- and self-pollen mixture treatment (Vaughton *et al*., 2010). No fruits were recorded in ten flowers pollinated with dead pollen, confirming that the pollen had been killed.

Pollen chase studies

The potential for ovule discounting was further investigated in *C. miniata* through a pollen chase experiment during the flowering season of 2012. Ten inflorescences from two stands (five inflorescences from each stand) of cultivated *C. miniata* plants in Pietermaritzburg were bagged as above. On opening, flowers were treated with: (1) self-pollen; (2) dead pollen; or (3) cross-pollen. Dead pollen was obtained and verified as for the ovule discounting experiment and cross-pollen was taken from discrete cultivated stands of *C. miniata* to avoid clonal or sibling mating. Cross-pollen was applied to each of the three treatments, 48 h subsequent to the initial treatments. Fruits were harvested 106 days later and scored.

EFFECTS OF POLLINATOR-MEDIATED SELF-POLLINATION ON FECUNDITY

To test whether natural fecundity in *C. miniata* and *C. gardenii* (robust form) is limited by pollinator-mediated self-pollination, fruit set and seed set (seeds per flower and seeds per fruit) of a single emasculated flower on an inflorescence (15 and seven plants for *C. miniata* and *C gardenii*, respectively) were compared with those of open controls (18 and 27 plants for *C. miniata* and *C. gardenii*, respectively) (Schoen & Lloyd, 1992; Eckert, 2000). To distinguish between effects of self-pollination within and between flowers (geitonogamy), these data were also compared with inflorescences which were entirely emasculated (14 plants for *C. miniata* and three plants for *C. gardenii*) (Schoen & Lloyd, 1992; Eckert, 2000). This was performed prior to anther dehiscence. Emasculation in these species is unlikely to affect pollinator visitation as *C. miniata* is pollinated by nectar-feeding butterflies, whereas *C. gardenii* is pollinated by nectar-feeding sunbirds (Kiepiel & Johnson, 2014).

To determine whether natural fecundity is limited by cross-pollination, fruit set and seed set were compared between naturally pollinated plants and those in which all available flowers received supplemental cross-pollination (Bierzychudek, 1981). This involved 13 supplemented and 14 control plants of *C. miniata* and seven supplemented and 27 control plants of *C. gardenii.* To establish the typical pollen loads on *C. miniata* stigmas and whether these were increased by supplemental hand-pollination, 13 open-pollinated and 11 cross-pollinated *C. miniata* flowers from the Karkloof site were examined. Pollen was counted by immersing stigmas in fuchsin gel on a glass slide sealed with a coverslip and examining them under a compound microscope at 40× magnification. To establish whether pollen loads on stigmas were sufficient to fertilize available ovules, the numbers of ovules in 147 flowers, each from a different plant, were counted.

STATISTICAL ANALYSIS

Data were analysed using generalized linear models implemented in SPSS 19.0 (IBM Corp.). Data for the proportion of flowers setting fruit and the proportion of germinated pollen grains that penetrated ovaries or ovules were analysed using models incorporating a binomial error distribution and logit link function. Data for the number of seeds per fruit, number of seeds per flower and number of ovules with visible pollen tube penetration were analysed using models that incorporated a negative binomial error distribution and log link function. To account for potential correlations among flowers on the same plant, we used generalized estimating equations with plant treated as the subject variable. These models employed an exchangeable correlation matrix with significance assessed using Score statistics. The exception was that, for data for seeds per fruit in the

Table 1. The mean (± SE) proportions of germinated pollen grains which reached the ovary and ovules after 48 h for 11 self-pollinated and four cross-pollinated flowers of *Clivia miniata* and 13 self-pollinated and 12 cross-pollinated flowers of *C. gardenii* (robust form)

	Ovary				Ovule			
	Treatment				Treatment			
	Cross	Self	χ^2	*P*	Cross	Self	χ^2	*P*
C. miniata	0.05 ± 0.02	0.03 ± 0.01	1.42	0.23	0.05 ± 0.02	0.01 ± 0.01	5.69	0.02
C. gardenii	0.30 ± 0.06	0.45 ± 0.08	2.40	0.12	0.14 ± 0.03	0.10 ± 0.03	0.98	0.32

pollen chase experiment, significance was assessed using Wald statistics, as Score statistics are known to be overly conservative when sample sizes are small (Guo *et al*., 2005). The sequential Šidák method was used to adjust for multiple comparisons. Marginal means were obtained by back-transformation of values from the linear scale, resulting in asymmetrical standard errors. In some cases in which generalized linear models did not run because of lack of variance (e.g. no fruits set in a treatment group), a single value was substituted, which also makes the test more conservative (Zuur *et al*., 2009).

RESULTS

Pollen tube analysis

Pollen tubes arising from germinated self- and cross-pollen grains of *C. miniata* and *C. gardenii* took about 48 h to enter the ovary (Figs 1E, F, 2). There was no statistically significant difference in the proportion of cross- and self-pollen tubes entering the ovary in *C. miniata* or *C. gardenii* (Table 1). Self-pollen tubes were significantly less likely than cross-pollen tubes to penetrate ovules in *C. miniata*, but there was no significant difference in the proportion of self- and cross-pollen tubes that penetrated ovules in *C. gardenii* (Table 1).

We were unable to compare ovule penetration of self- and cross-pollinated pistils beyond 48 h, as we examined only cross-pollinated pistils of *C. miniata* and self-pollinated pistils of *C. gardenii* after 72 h. Ovule penetration appears to continue after 48 h, at least in *C. gardenii*. For self-pollinated pistils of *C. gardenii*, the mean number of ovules per ovary in which pollen tube penetration was evident was 5.50 [lower standard error (SE), 1.39; upper SE, 1.86] after 72 h compared with 1.69 (lower SE, 0.50; upper SE, 0.71) after 48 h ($\chi^2 = 6.618$; $P = 0.010$). For cross-pollinated pistils of *C. miniata*, the mean number of ovules per ovary in which pollen tube penetration was evident was 3.79 (lower SE, 0.99; upper SE, 1.32) after 72 h compared with 2.25 (lower SE, 1.02; upper SE, 1.85) after 48 h ($\chi^2 = 0.574$; $P = 0.449$).

Breeding system

Few of the bagged and unmanipulated flowers of *C. miniata* and *C. gardenii* set fruit (Fig. 3), indicating that these species are not autogamous. Self-pollination in both species yielded similarly low levels of fruit set (< 10% of flowers), whereas, on average, about 50% of cross-pollinated flowers set fruit (Fig. 3). There were no significant differences in the number of seeds per fruit in the various treatments, but the power to detect differences for this particular measure of fecundity was low because of the small number of fruits arising in the bagged unmanipulated and self-pollination treatments. The overall number of seeds produced by cross-pollinated flowers was significantly higher than for bagged unmanipulated and self-pollinated flowers (Fig. 3).

Potential for ovule discounting

Pollen mixture studies

In the controlled hand-pollination experiments involving the application of pollen mixtures to stigmas of *C. miniata* at the Karkloof site and *C. gardenii* at the Pietermaritzburg site, fruit set in cross-pollinated flowers was significantly greater than that in self-pollinated flowers (Fig. 4), thus reinforcing the results obtained in earlier experiments (Fig. 3). Relative to the application of pure cross-pollen, application of a mixture of self- and cross-pollen led to significant reductions in fruit set in *C. gardenii*, seeds per fruit in both *C. miniata* and *C. gardenia*, and seeds per flower in *C. gardenii* (Fig. 4). However, the application of a mixture of dead self- and live cross-pollen did not yield results which were significantly different from those obtained when a mixture of live self- and live cross-pollen was applied (Fig. 4).

Pollen chase studies

There was no significant difference in fruit set or seeds per flower between the three treatments

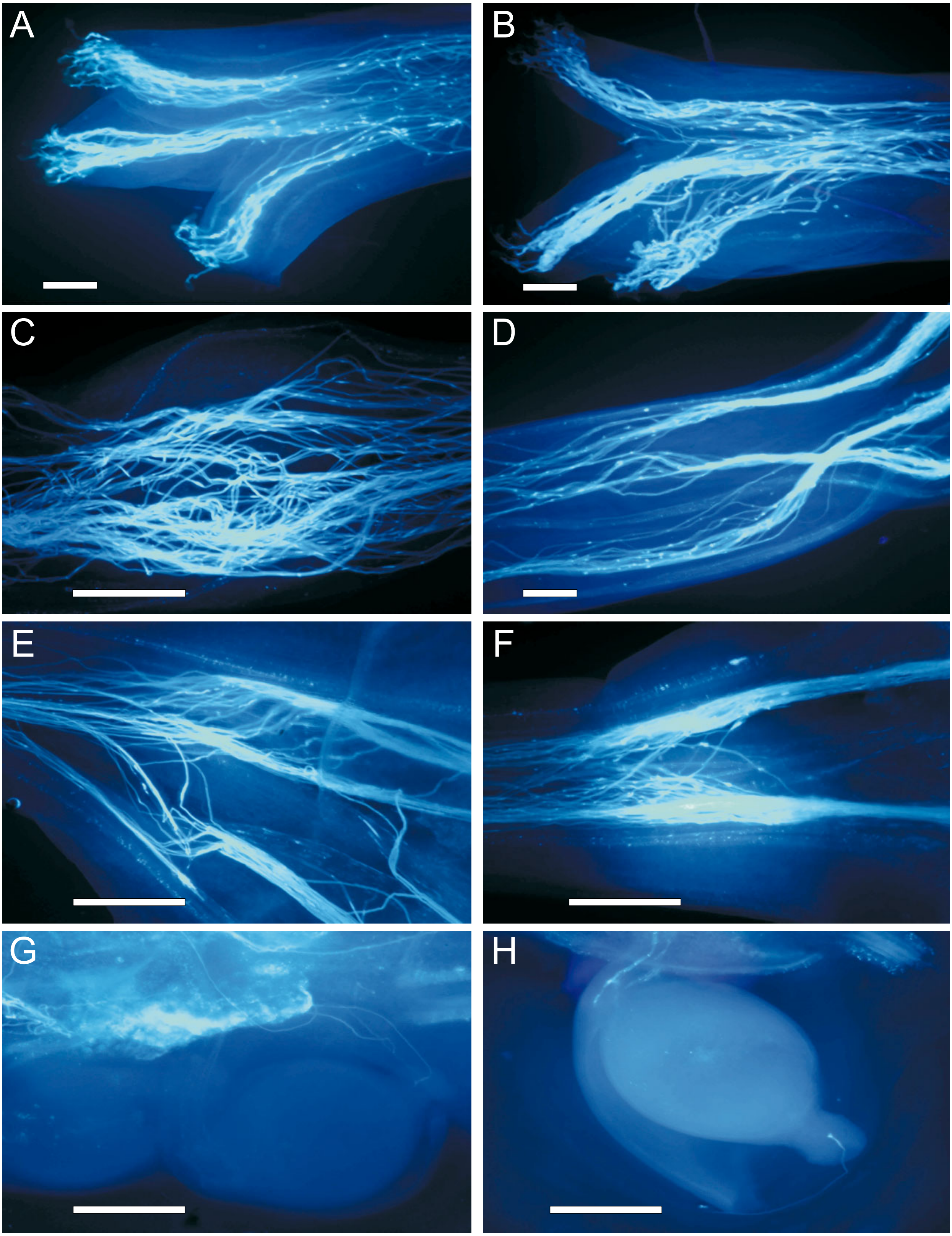

Figure 1. Development of cross- (left panels) and self- (right panels) pollen tubes in *Clivia miniata*. Pollen tubes are visible growing into the stigma tip (A, B), in the mid-style of the pistil (C, D), at the top of the ovaries (E, F) and penetrating individual ovules (G, H). Scale bars, 50 μm.

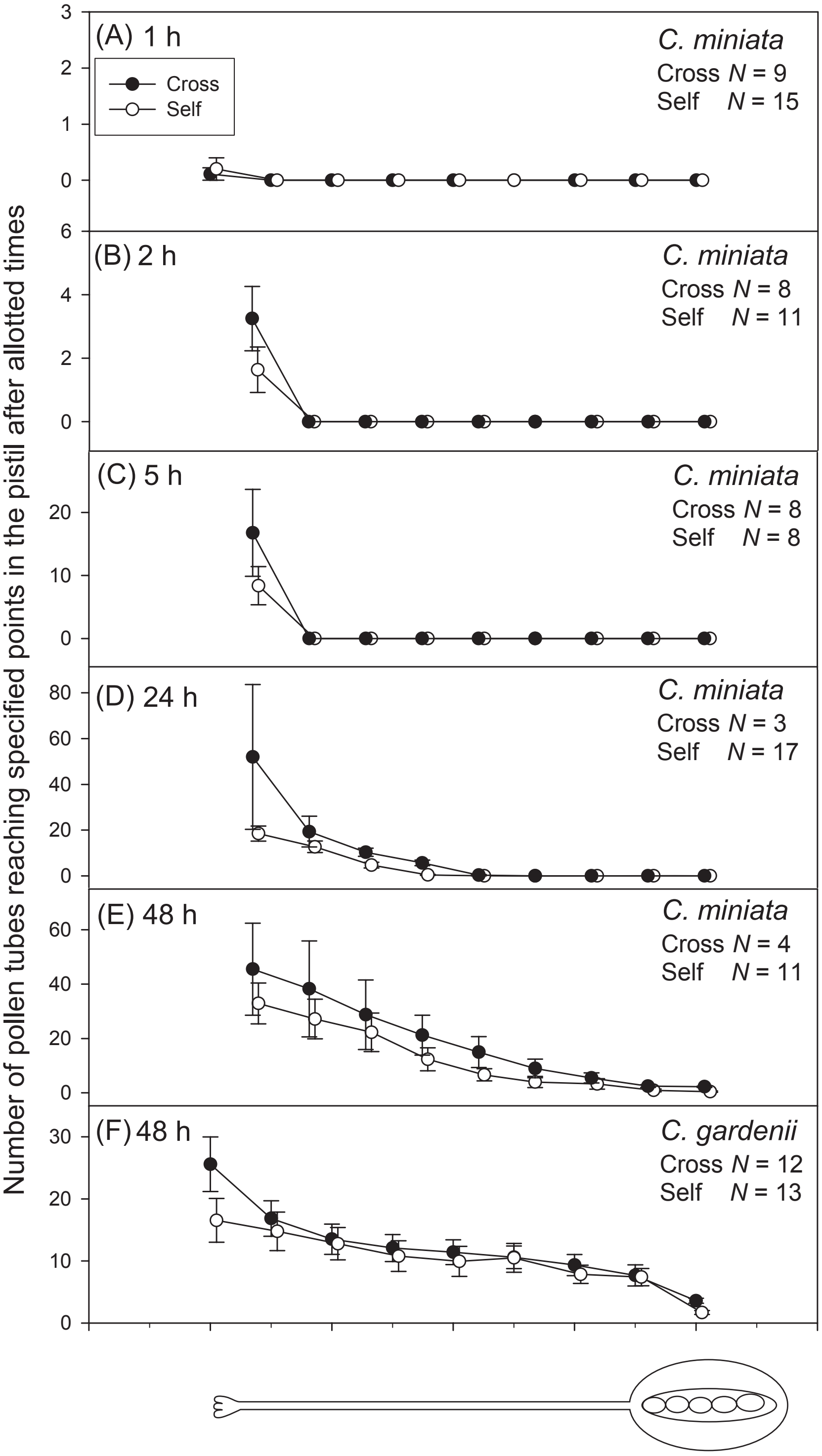

Figure 2. Proportion of cross- and self-pollen tubes (mean ± SE) reaching various landmarks in the pistil (illustrated below the *y* axis) of *Clivia miniata* (A–E) and *C. gardenii* (F) at various time periods after pollination. Sample size (*N*) represents the number of stigmas, each of which was taken from a different plant.

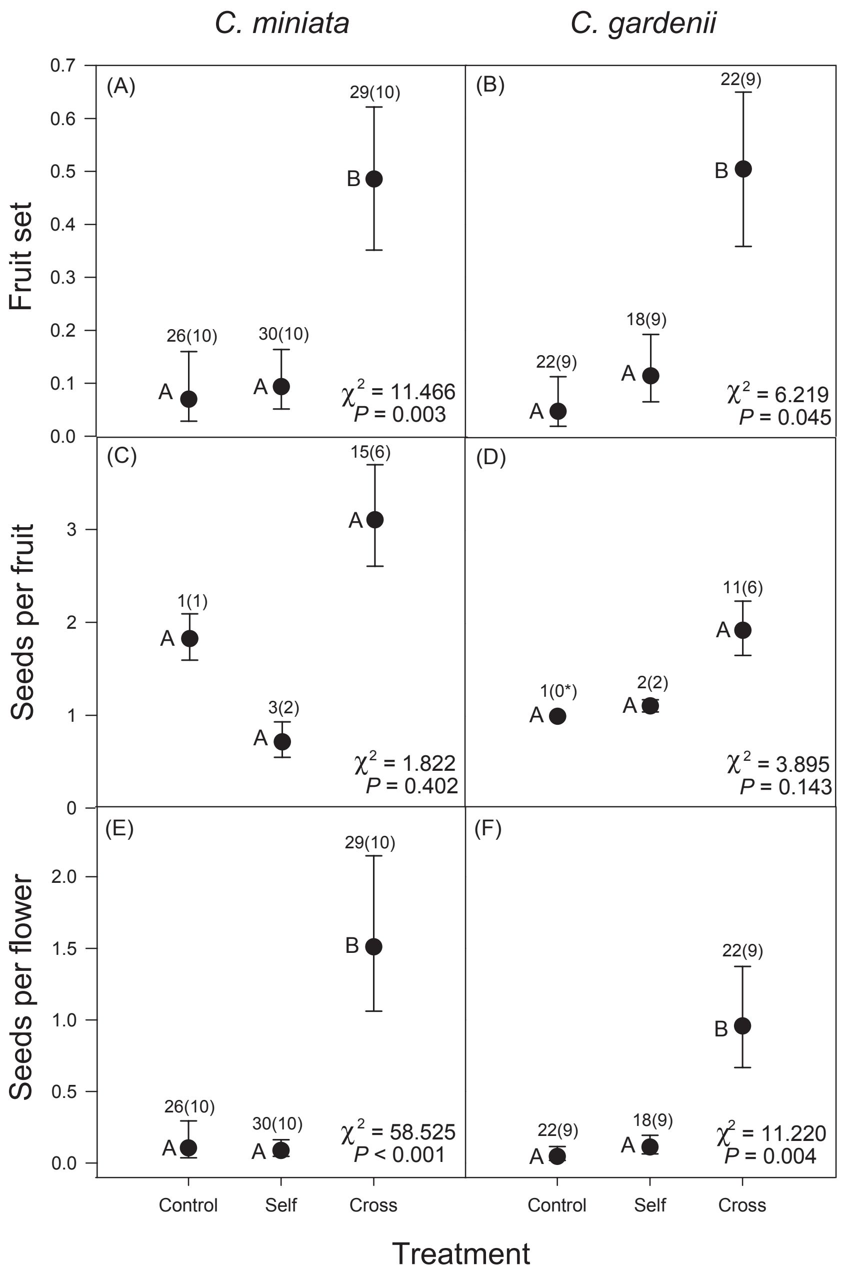

Figure 3. The effects of controlled hand pollinations to determine the breeding systems of *Clivia miniata* (left panels) and *C. gardenii* (right panels). Values are mean (± SE) proportion of flowers setting fruit (A, B), number of seeds per fruit (C, D) and number of seeds per flower (E, F). Controls refer to flowers that were bagged without manipulation. Sample sizes are flowers with number of plants given in parentheses. Means that share the same letters are not significantly different (Dunn–Šidák test).

involving pollination with different mixtures prior to cross-pollination (Fig. 5). However, prior pollination with self-pollen or dead self-pollen both resulted in significantly fewer seeds per fruit than did prior pollination with cross-pollen (Fig. 5).

EFFECTS OF POLLINATOR-MEDIATED SELF-POLLINATION ON FECUNDITY

In both *C. miniata* and *C. gardenii*, neither emasculation of single flowers to prevent within-flower self-pollination nor emasculation of all flowers

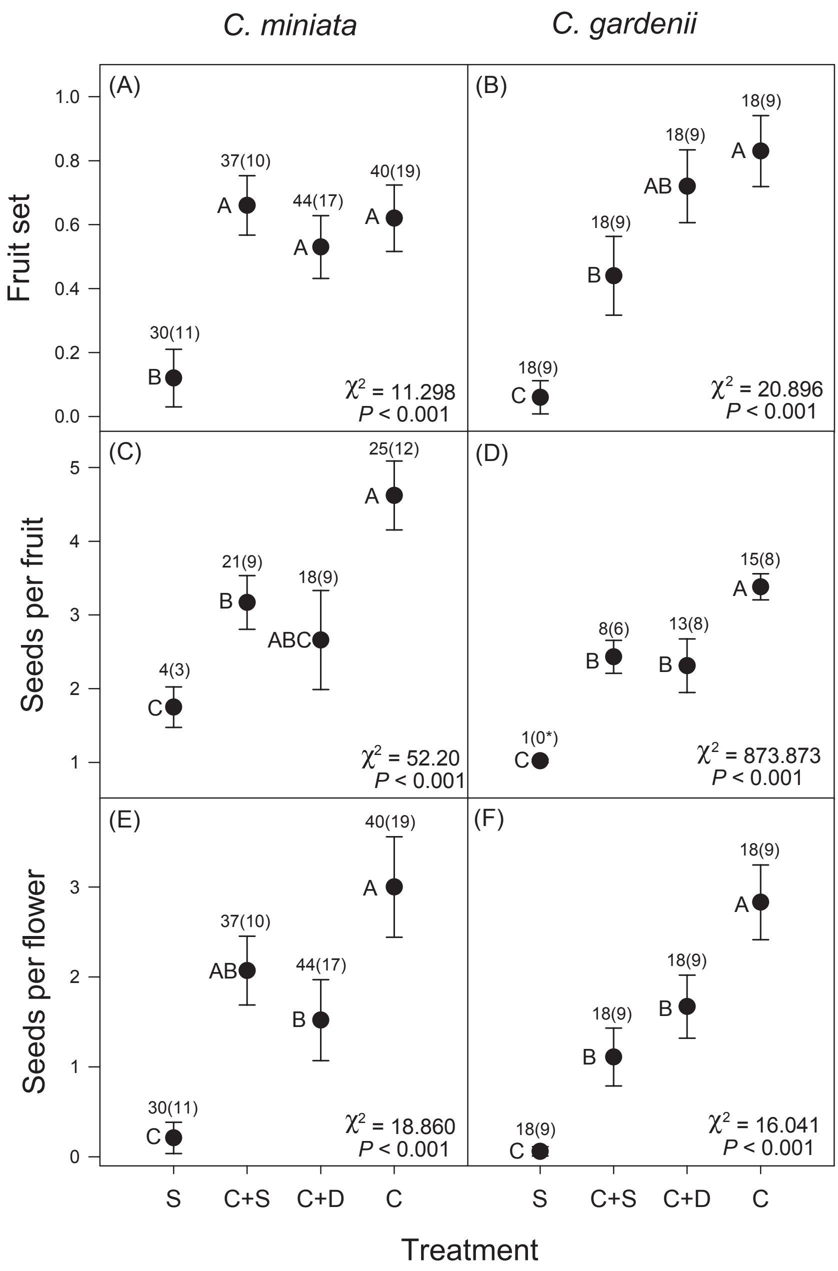

Figure 4. The effects of application of various pollen mixtures to determine the potential for ovule discounting in *Clivia miniata* (left panels) and *C. gardenii* (right panels). Values are mean (± SE) proportions of flowers setting fruit (A, B), number of seeds per fruit (C, D) and number of seeds per flower (E, F). Treatment abbreviations: S, self-pollen; C + S, cross- + self-pollen; C + D, cross- + dead pollen; C, cross-pollen. Sample sizes are flowers with number of plants given in parentheses. Means that share the same letter are not significantly different (Dunn–Šidák test).

on inflorescences to prevent geitonogamous self-pollination had an effect on fecundity (Fig. 6). There were also no significant effects of pollen supplementation on fruit set, seeds per fruit or seeds per flower in either species (Fig. 6). Stigmas of naturally pollinated *C. miniata* flowers had, on average (± SE), 100.5 ± 9.26 grains, whereas those supplemented with pollen had 278.6 ± 11.38 grains ($t = 17.94$, d.f. = 2, $P < 0.001$). The mean number of pollen grains found on naturally pollinated *C. miniata* stigmas was about six times greater than the mean number of available ovules (16.4 ± 0.18).

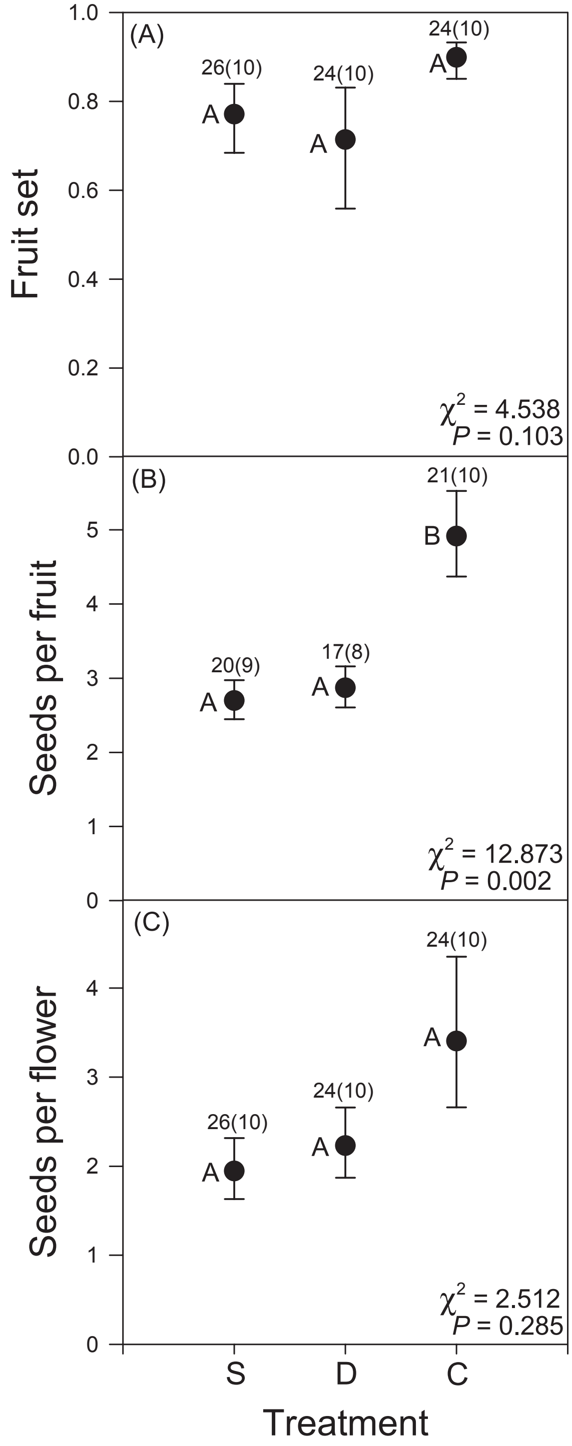

Figure 5. The effects of the prior application of self-pollen (S), dead pollen (D) or cross-pollen (C) on fecundity of subsequently cross-pollinated flowers of *Clivia miniata*. Values are mean (± SE) proportions of flowers setting fruit (A), number of seeds per fruit (B) and number of seeds per flower (C). Sample sizes are flowers with number of plants given in parentheses. Means that share the same letter are not significantly different (Dunn–Šidák test).

DISCUSSION

The results of this study indicate that *C. miniata* and *C. gardenii* are both effectively self-sterile and therefore reliant on vectors for seed production (Figs 3, 4). Both species are nevertheless capable of the production of small amounts of seed through self-fertilization (Fig. 3). These data are consistent with anecdotal reports for this species, ranging from the production of small amounts of seed following self-pollination (Duncan, 1999; van der Merwe *et al.*, 2005; Swanevelder & Fisher, 2009) to complete self-sterility in some plants (Swanevelder & Fisher, 2009). It is likely that reports of the production of viable seed through selfing in horticulture refer either to this limited capacity or to plants in lines that have been selected for self-fertilization ability.

Pollen tubes from germinated self-pollen were as likely as those from germinated cross-pollen to reach the ovaries in *C. miniata* and both the ovaries and ovules in *C. gardenii* (Table 1; Fig. 2). This rules out a conventional gametophytic SI system acting in the style, and, when considered in the light of the low seed set obtained from self-pollination, is consistent with the presence of either an LSI system or severe early inbreeding depression. Recent studies on *Aconitum kusnezoffii* Rchb. (Ranunculaceae) have indicated that pre-zygotic LSI may work in combination with early-acting inbreeding depression, with the former responsible for the partial rejection of self-pollen tubes and the latter terminating a component of seeds which pre-zygotic LSI was unable to prevent (Hao *et al.*, 2012).

Unlike our pollen tube studies, experiments in which different mixtures of self- and cross-pollen were applied to stigmas of *C. miniata* and *C. gardenii* did not provide clear-cut evidence for LSI. In experiments of this kind conducted on plants with LSI, it would be expected that seed production would be compromised through ovule discounting if self-pollen is applied together with cross-pollen (Waser & Price, 1991; Broyles & Wyatt, 1993; Barrett *et al.*, 1996; Sage *et al.*, 1999, 2006; Vaughton *et al.*, 2010). This was partially evident from the significant reduction in the number of seeds per fruit in flowers that received mixtures of self- and cross-pollen (Fig. 4B), but flowers that received mixtures of cross- and dead pollen did not set more seed than those that received mixtures of viable cross- and self-pollen (Fig. 4). Similarly, the application of dead pollen prior to cross-pollen appeared to inhibit seed set relative to the application of pure cross-pollination (Fig. 5). It is therefore uncertain whether the depression of seed set in flowers that receive mixtures of self- and cross-pollen (Fig. 4), or self-pollen before cross-pollen (Fig. 5), is a result of ovule discounting or other

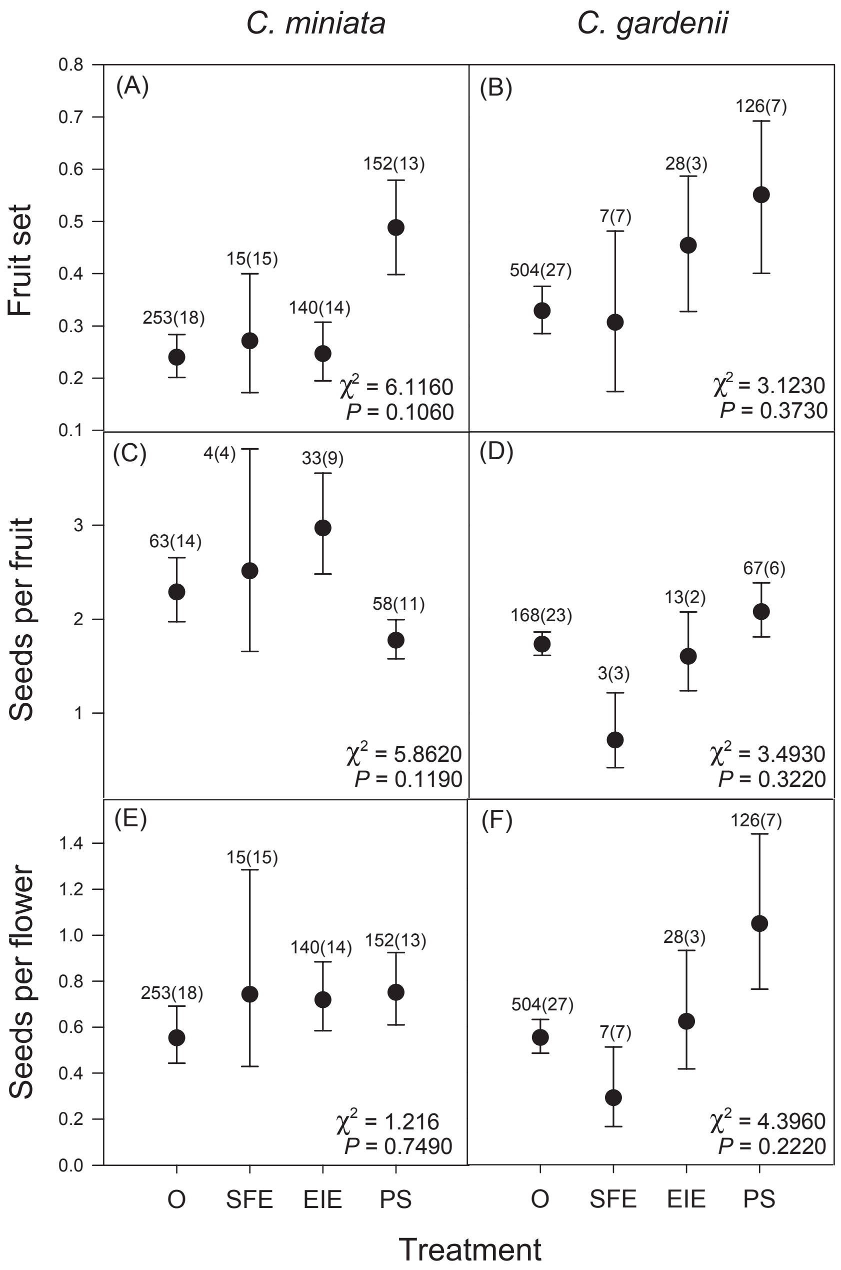

Figure 6. The effects of emasculation and pollen supplementation on the fecundity of *Clivia miniata* (left panels) and *C. gardenii* (right panels). Values are mean (± SE) proportions of flowers setting fruit (A, B), number of seeds per fruit (C, D) and number of seeds per flower (E, F). Abbreviations: O, open control; SFE, single flower emasculations; EIE, entire inflorescence emasculations; PS, pollen supplemented. Sample sizes are flowers with number of plants given in parentheses.

interactions on the stigma, perhaps involving chemical factors from self-pollen that are not destroyed by microwaving, a chemical artefact of the microwaving itself or physical blocking of cross-pollen by dead pollen on the stigma surface. In their study of another amaryllid, *Cyrtanthus breviflorus*, Vaughton *et al.* (2010) found that the application of a mixture of cross-pollen and dead pollen yielded as much seed as did the application of pure live cross-pollen, but one difference is that they used dead cross-pollen in their experiments, whereas we applied dead self-pollen in mixtures.

It is difficult to exclude entirely the possibility that *Clivia* plants are self-compatible with high levels of early inbreeding depression. It has been argued that consistently low (< 10%) fruit set and minimal seed production in self-pollinated flowers, as recorded in most of our experiments with *Clivia*, is indicative of LSI rather than inbreeding depression (Seavey & Bawa, 1986). SI systems can also be leaky, particularly in Amaryllidaceae (Barrett *et al*., 1997). In *Cyrtanthus breviflorus* which was inferred to possess LSI, up to 40% of self-pollinated flowers produced fruit, but with few seeds (Vaughton *et al*., 2010). Further evidence for LSI in *C. miniata* and *C. gardenii* is that the majority of hand self-pollinated flowers abscise in the same time span (approximately 1 week) that it takes unpollinated senescing flowers to abscise, which is not a pattern that would be expected from early inbreeding depression (Seavey & Bawa, 1986).

FACTORS LIMITING SEED PRODUCTION IN *CLIVIA* POPULATIONS

Populations of *Clivia* spp. are characterized by low levels of fruit set and the development of seeds in a small proportion of the available ovules (Figs 4, 5). Our data indicate that levels of natural fruit and seed set in wild populations are far lower than they are in cultivated plants (van der Merwe *et al*., 2005; Swanevelder & Fisher, 2009).

Possible explanations for low natural fecundity include resource limitation (Charlesworth, 1989), inadequate cross-pollination (Burd, 1994) and ovule discounting arising from self-pollination (Waser & Price, 1991; Sage *et al*., 1999; Vaughton & Ramsey, 2010; Vaughton *et al*., 2010). For example, it has been found that, in the self-incompatible amaryllid *Narcissus cyclamineus* Redouté, self-pollination prior to cross-pollination causes ovule discounting through loss of viable ovules (Navarro *et al*., 2012). As the emasculation of flowers in natural populations of *C. miniata* and *C. gardenii* did not affect seed production significantly (Fig. 6), it also seems that natural fecundity is not limited by ovule discounting arising through pollinator-mediated self-pollination. In the case of *C. miniata*, we observed that butterflies seldom visit more than one flower per plant (Kiepiel & Johnson, 2014), thus reducing the likelihood of geitonogamous self-pollination.

Supplemental application of cross-pollen did not increase seed production significantly in populations of the two *Clivia* spp. (Fig. 6), indicating that fecundity in these plants is not limited by the quantity or quality of pollination (Burd, 1994; Harder & Aizen, 2010). On average, plants in wild populations, even when hand cross-pollinated, failed to set fruits in > 50% of their flowers (Fig. 6). In contrast, almost all cross-pollinated flowers of cultivated *C. gardenii* plants set fruit (Fig. 4). Therefore, resource limitation seems to be the most likely explanation for low fruit and seed production in natural *Clivia* populations. We found that the number of *C. miniata* pollen grains on open-pollinated stigmas in the Umtamvuna population was about five-fold greater than the mean number of available ovules, but, on average, only one in five ovules developed into seeds. Low seed production in *Clivia* may be explained by resource trade-offs involved in the development of the large, fleshy, recalcitrant seeds. This is reflected in the long seed development (up to 15 months in *C. gardenii*; van der Merwe *et al*., 2005) and the fact that some individuals flower and set seed at intervals of several years rather than annually. It has been suggested that, in outbreeding species, which are long lived, low female fecundity has developed as a response to high maternal investment in seed production (Charlesworth, 1989).

CONCLUSIONS

This study shows that two *Clivia* spp. are effectively self-sterile and thus reliant on pollinators for reproduction. This, considered together with our studies of pollen tube development, suggests that *Clivia* has LSI, as reported for other tribes of Amaryllidaceae, rather than a classical gametophytic SI system acting in the style. However, it is not possible to exclude early inbreeding depression as an alternative explanation for these results. Emasculation experiments did not provide support for the hypothesis that low levels of natural seed production in *Clivia* are the result of ovule discounting. Instead, it appears that the production of the large fleshy seeds is mainly resource limited. Further histological and genetic studies are required to unequivocally confirm or reject LSI as the underlying basis of the observed late-acting self-sterility in *Clivia*.

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REFERENCES

Allen AM, Hiscock SJ. 2008. Evolution and phylogeny of self-incompatibility systems in angiosperms. In: Franklin-Tong VE, ed. *Self-incompatibility in flowering plants – evolution, diversity and mechanisms*. Berlin: Springer, 73–101.

Barrett SCH, Cole WW, Arroyo J, Cruzan MB, Lloyd DG. 1997. Sexual polymorphisms in *Narcissus triandrus* (Amaryllidaceae): is this species tristylous? *Heredity* **78:** 135–145.

Barrett SCH, Lloyd DG, Arroyo J. 1996. Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). In: Lloyd DG, Barrett SCH, eds. *Floral biology: studies on floral evolution in animal pollinated plants*. New York: Chapman and Hall, 339–376.

Bateman AJ. 1954. The genetics of *Narcissus*. I. Sterility. In: Synge PM, ed. *Daffodil and Tulip Year Book 19*. London: Royal Horticultural Society, 23–29.

Beardsell DV, Knox RB, Williams EG. 1993. Breeding system and reproductive success of *Thryptomene calycina* (Myrtaceae). *Australian Journal of Botany* **41:** 333–353.

Bierzychudek P. 1981. Pollinator limitation of plant reproductive effort. *American Naturalist* **117:** 838–840.

Bittencourt NS, Gibbs PE, Semir J. 2003. Histological study of post-pollination events in *Spathodea campanulata* Beauv. (Bignoniaceae), a species with late-acting self-incompatibility. *Annals of Botany* **91:** 827–834.

Bittencourt NS, Semir J. 2005. Late-acting self-incompatibility and other breeding systems in *Tabebuia* (Bignoniaceae). *International Journal of Plant Sciences* **166:** 493–506.

Brewbaker JL. 1959. Biology of the angiosperm pollen grain. *Indian Journal of Genetics and Plant Breeding* **19:** 121–133.

Broyles SB, Wyatt R. 1993. The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *American Journal of Botany* **80:** 41–44.

Burd M. 1994. Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review* **60:** 83–139.

Charlesworth D. 1985. Distribution of dioecy and self-incompatibility in angiosperms. In: Greenwood PJ, Harvey PH, Slatkin M, eds. *Evolution: essays in honour of John Maynard Smith*. Cambridge: Cambridge University Press, 237–268.

Charlesworth D. 1989. Evolution of low female fertility in plants: pollen limitation, resource allocation and genetic load. *Trends in Ecology & Evolution* **4:** 289–292.

Chen X, Hao S, Wang L, Fang W, Wang Y, Li X. 2012. Late-acting self-incompatibility in tea plant (*Camellia sinensis)*. *Biologia* **67:** 347–351.

Conrad F. 2008. Molecular systematics, biogeography and dating of the tribe Haemantheae (Amaryllidaceae) and the phylogeography of *Clivia*. DPhil Thesis, University of Cape Town.

Cope FW. 1962. The mechanism of self-incompatibility in *Theobroma cacao* L. *Heredity* **17:** 157–182.

Cruzan MB. 1986. Pollen tube distributions in *Nicotiana glauca:* evidence for density dependent growth. *American Journal of Botany* **73:** 902–907.

Darlington CD, Mather K. 1949. *The elements of genetics*. London: Allen & Unwin.

Dulberger R. 1964. Flower dimorphism and self-incompatibility in *Narcissus tazetta* L. *Evolution* **18:** 361–363.

Duncan GD. 1999. G*row clivias*. Cape Town: National Botanical Institute.

Eckert CG. 2000. Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology* **81:** 532–542.

Ford CS, Wilkinson MJ. 2012. Confocal observations of late-acting self-incompatibility in *Theobroma cacao* L. *Sexual Plant Reproduction* **25:** 169–183.

Gibbs PE, Bianchi M. 1993. Post-pollination events in species of *Chorisia* (Bombacaceae) and *Tabebuia* (Bignoniaceae) with late-acting self-incompatibility. *Botanica Acta* **106:** 64–71.

Gibbs PE, Bianchi MA. 1999. Does late-acting self-incompatibility (LSI) show family clustering? Two more species of Bignoniaceae with LSI: *Dolichandra cynanchoides* and *Tabebuia nodosa*. *Annals of Botany* **84:** 449–457.

Gibbs PE, Oliveira PE, Bianchi MB. 1999. Post-zygotic control of selfing *Hymenaea stigonocarpa* (Leguminosae–Caesalpinioideae), a bat-pollinated tree of the Brazilian cerrados. *International Journal of Plant Sciences* **160:** 72–78.

Guo X, Pan W, Connett JE, Hannan PJ, French SA. 2005. Small-sample performance of the robust score test and its modifications in generalized estimating equations. *Statistics in Medicine* **24:** 3479–3495.

Hao YQ, Zhao XF, She DY, Xu B, Zhang DY, Liao WJ. 2012. The role of late-acting self-incompatibility and early-acting inbreeding depression in governing female fertility in monkshood, *Aconitum kusnezoffii*. *Plos One* **7:** 1–7.

Harder LD, Aizen MA. 2010. Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society Series B* **365:** 529–543.

Herlihy CR, Eckert CG. 2002. Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* **416:** 320–323.

Hinata K, Isogai A, Isuzugawa K. 1994. Manipulation of sporophytic self-incompatibility in plant breeding. In: Williams EG, Clarke AE, Knox RB, eds. *Genetic control of self-incompatibility and reproductive development in flowering plants*. Dordrecht: Kluwer Academic Publishers, 102–115.

Kenrick J, Kaul V, Williams EG. 1986. Self-incompatibility in *Acacia retinodes:* site of pollen-tube arrest is the nucellus. *Planta* **169:** 245–250.

Kiepiel I, Johnson SD. 2014. Shift from bird to butterfly pollination in Clivia (Amaryllidaceae). *American Journal of Botany* **101:** 190–200.

Knight R, Rogers HH. 1955. Incompatibility in *Theobroma cacao*. *Heredity* **9:** 66–77.

Koopowitz H. 2002. *Clivias*. Portland, OR: Timber Press.

LaDoux T, Friar EA. 2006. Late-acting self-incompatibility in *Ipomopsis tenuifolia* (Gray) V. Grant (Polemoniaceae). *International Journal of Plant Sciences* **167:** 463–471.

Lipow SR, Wyatt R. 2000. Single gene control of post-zygotic self-incompatibility in poke milkweed, *Asclepias exaltata* L. *Genetics* **154:** 893–907.

Lloyd DG. 1992. Self- and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences* **153:** 370–380.

Lloyd DG, Wells MS. 1992. Reproductive biology of a primitive angiosperm, *Pseudowintera colorata* (Winteraceae), and the evolution of pollination systems in the Anthophyta. *Plant Systematics and Evolution* **181:** 77–95.

Manning J. 2005. Pollination in *Clivia*. In: Dixon R, Felbert C, van der Linde J, eds. *Clivia seven*. Cape Town: Clivia Society, 17–22.

Martin FW. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* **34:** 125–128.

Meerow AW, Fay MF, Guy CL, Li Q-B, Zaman FQ, Chase MW. 1999. Systematics of Amaryllidaceae based on cladistic analysis of plastid sequence data. *American Journal of Botany* **86:** 1325–1345.

van der Merwe L, Robbertse H, de Kock B. 2005. *Cultivation of clivias*. Pretoria: Clivia Society.

Murray BG, Ran Y, De Lange PJ, Hammett KRW, Truter JT, Swanevelder ZH. 2004. A new species of *Clivia* (Amaryllidaceae) endemic to the Pondoland Centre of Endemism, South Africa. *Botanical Journal of the Linnean Society* **146:** 369–374.

Naaborgh AT, Willemse MTM. 1991. The ovular incompatibility system in *Gasteria verrucosa* (Mill.) H. Duval. *Euphytica* **58:** 231–240.

Navarro L, Ayensa G, Ferrero V, Sanchez JM. 2012. The avoidance of self-interference in the endemic daffodil *Narcissus cyclamineus* (Amaryllidaceae). *Plant Ecology* **213:** 1813–1822.

de Nettancourt D. 1977. *Incompatibility in angiosperms*. Berlin: Springer-Verlag.

de Nettancourt D. 1997. Incompatibility in angiosperms. *Sexual Plant Reproduction* **10:** 185–199.

Pandey KK. 1960. Evolution of gametophytic and sporophytic systems of self-incompatibility in angiosperms. *Evolution* **14:** 98–115.

Rourke JP. 2002. *Clivia mirabilis* (Amaryllidaceae: Haemantheae) a new species form the Northern Cape, South Africa. *Bothalia* **32:** 1–7.

Sage TL, Bertin RI, Williams EG. 1994. Ovarian and other late-acting self-incompatibility systems. In: Williams EG, Clarke AE, Knox RB, eds. *Genetic control of self-incompatibility and reproductive development in flowering plants*. Dordrecht: Kluwer Academic Publishers, 116–140.

Sage TL, Price MV, Waser NM. 2006. Self-sterility in *Ipomopsis aggregata* (Polemoniaceae) is due to prezygotic ovule degeneration. *American Journal of Botany* **93:** 254–262.

Sage TL, Sampson FB. 2003. Evidence for ovarian self-incompatibility as a cause of self-sterility in the relictual woody angiosperm, *Pseudowintera axillaris* (Winteraceae). *Annals of Botany* **91:** 807–816.

Sage TL, Strumas F, Cole WW, Barrett SCH. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* **86:** 855–870.

Sage TL, Williams EG. 1991. Self-incompatibility in *Asclepias*. *Plant Cell Incompatibility Newsletter* **23:** 55–57.

Schoen DJ, Lloyd DG. 1992. Self- and cross-fertilization in plants. III. Methods for studying modes and functional aspects of self-fertilization. *International Journal of Plant Sciences* **153:** 381–393.

Sears ER. 1937. Cytological phenomena connected with self-sterility in the flowering plants. *Genetics* **22:** 130–181.

Seavey SR, Bawa KS. 1986. Late-acting self-incompatibility in angiosperms. *Botanical Review* **52:** 195–219.

Swanevelder D, Fisher RC. 2009. Clivia: *nature and nurture*. Pretoria: Briza Publications.

Swanevelder ZH. 2003. Diversity and population structure of *Clivia miniata* Lindl. (Amaryllidaceae): evidence from molecular genetics and ecology. MSc Dissertation. Bloemfontein: University of the Free State.

Van Wyk AE. 1994. Maputaland–Pondoland Region. In: Davis SD, Heywood VH, Hamilton AC, eds. *Centres of plant diversity. A guide and strategy for their conservation, Vol. 1*. Cambridge: IUCN Publications Unit, pp. 227–235.

Van Wyk AE, Smith GF. 2001. *Regions of floristic endemism in southern Africa. A review with emphasis on succulents*. Pretoria: Umdaus Press.

Vaughton G, Ramsey M. 2010. Floral emasculation reveals pollen quality limitation of seed output in *Bulbine bulbosa* (Asphodelaceae). *American Journal of Botany* **91:** 174–178.

Vaughton G, Ramsey M, Johnson SD. 2010. Pollination and late-acting self-incompatibility in *Cyrtanthus breviflorus* (Amaryllidaceae): implications for seed production. *Annals of Botany* **106:** 547–555.

Waser NM, Price MV. 1991. Reproductive costs of self-pollination in *Ipomopsis aggregata* (Polemoniaceae): are ovules usurped? *American Journal of Botany* **78:** 1036–1043.

Winter J. 2000. The natural distribution and ecology of *Clivia*. In: Dower M, Felbert C, Winter J, eds. *Clivia Yearbook 2*. Cape Town: Kirstenbosch National Botanical Gardens, 5–9.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. *Mixed model effects and extensions in ecology with R*. New York: Springer.

CHAPTER 4

FUNCTIONS OF FLORAL TRAITS IN THE MUTUALISM BETWEEN THE BUSH LILY *CLIVIA MINIATA* (AMARYLLIDACEAE) AND SWALLOWTAIL BUTTERFLIES

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- *Premise of study*: Butterflies are important pollinators for many plants, but the floral traits that mediate these interactions are not well understood from a functional perspective, particularly under field conditions. We examined the functional significance of floral traits in *Clivia miniata* (Amaryllidaceae), a southern African forest lily that is pollinated largely by swallowtail butterflies and evolved in a lineage in which bird pollination was ancestral.
- *Methods:* Floral spectral variation was assessed in *C. miniata* and the sympatric bird-pollinated congener *C. gardenii*. We used arrays in the field to establish whether butterflies prefer the common *C. miniata* var. *miniata* (orange flowers) over the rare *C. miniata* var. *citrina* (yellow flowers). Model (artificial) flowers varying in colour, colour patterning, shape and size were used to further assess cues that are used by butterflies. Arrays of upright and pendulous *C. miniata* flowers were set up to determine the effects of floral orientation on butterfly visitation. Scent-supplemented flower arrays (model and real) and electroantennographic detection was used to determine butterfly response to scent of *C. miniata* flowers.
- *Key results:* Butterflies preferred the orange morph of *C. miniata* over the yellow morph. Butterflies preferred red and orange over yellow model flowers. They also preferred orange models with a yellow target “nectar guide” over plain orange models. Butterflies preferred to alight on flowers in an upright position and favoured larger over smaller model flowers. The addition of scent to model and actual flowers increased butterfly visitation. Swallowtail butterflies showed weak antennal responses to several compounds in the floral scent of *C. miniata*.
- *Conclusions:* Colour is a primary advertising cue for butterflies, working synergistically with other floral signals. Butterflies show hierarchical behavioural responses according to colour, colour pattern, shape and scent signals which approximate those found in flowers of *C. miniata*. Manipulation of floral orientation from upright to pendulous, resulted in floral isolation. These results shed light on the cues used by foraging swallowtail butterflies and contribute to the understanding of floral evolution during shifts from bird to butterfly pollination.

Keywords: Advertising signals; colour preferences; floral evolution; floral traits; Lepidoptera; flower orientation; pollination; pollinator behaviour; scent

INTRODUCTION

Pollinator-mediated selection on floral traits is considered a fundamental element of angiosperm evolutionary diversification (Darwin, 1862; Stebbins, 1970; Faegri and van der Pijl, 1979; Harder and Johnson, 2009). Patterns of convergence in suites of floral traits among unrelated taxa sharing pollinator groups are considered to reflect underlying adaptations to sensory preferences, morphology and behaviour of pollinators, and are termed pollination or floral syndromes (Faegri and van der Pijl, 1979). The syndrome concept assumes some degree of specialization of pollination systems at a functional group level, defining for example bird or butterfly-pollinated flowers. While macroevolutionary tests of associations between suites of traits and pollinators have been used successfully to test the concept of floral syndromes (Van der Niet and Johnson, 2012; Johnson and Wester, 2017), detailed experimental studies of trait selection by pollinators are required to understand the mechanistic basis of floral trait evolution. Studies of phenotypic selection (Sletvold and Ågren, 2010; Sletvold et al., 2016; Caruso et al., 2019) and experimental evolution (Gervasi and Schiestl, 2017) are valuable in this regard, but in some cases it is necessary to reintroduce phenotypic variation that may have been eliminated by selection or to use model (artificial flowers) or manipulated (real flowers) to gain a mechanistic understanding (Clements and Long, 1923; Meléndez-Ackerman, Campbell, and Waser, 1997; Campbell, 2009; Pohl, Van Wyk, and Campbell, 2011; Jersáková et al., 2012; Campbell, Jürgens, and Johnson, 2016). Experimental choice arrays using model or manipulated flowers provide tools for identifying the floral traits responsible for pollinator attraction, allowing for the systematic isolation of the individual cues responsible for generating advertising signals and the precise evaluation of the relative strength of these signals (Clements and Long, 1923; Bell, 1985; Mitchell-Olds and Shaw, 1987; Lunau, 1990; Schemske and Ågren, 1995; Johnson and Dafni, 1998; Campbell, 2009; Jersáková et al., 2012; Kaczorowski et al., 2012; Campos, Bradshaw, and Daniel, 2015; Policha et al., 2016; Hirota et al., 2019).

Plants with biotic pollination systems rely heavily on animal attraction for fitness (Darwin, 1877; Waser, 1983) and floral traits that increase the visitation frequency of pollinators are expected to be favoured by selection. Pollinators can display marked preferences towards particular floral traits (Grant, 1949; Grant and Grant, 1965; Waser, 1983; Waser and Price, 1983; Hodges and Arnold, 1994b; Waser, 1998; Fulton and Hodges, 1999; Bradshaw and Schemske, 2003; Campbell, Jürgens, and Johnson, 2016). Variation in floral traits such as colour, size, shape, orientation, scent or nectar production, can thus influence plant fitness through the alteration of pollinator visitation rates (Campbell, 1989; Campbell, Waser, and Meléndez-Ackerman, 1997; Dafni and Kevan, 1997; Schemske and Bradshaw, 1999; Campbell, Jürgens, and Johnson, 2016). The perception and sensory bias of pollinators to particular floral traits therefore plays a fundamental role in floral evolution (Schiestl and Dötterl, 2012). The innate preferences pollinators have for certain floral traits, tailors their behavioural biases and allows for the identification of flower rewards, whilst providing the learning flexibility required for associative learning of new advertising cues (Gould and Marler, 1984; Lunau and Maier, 1995). Understanding floral evolution therefore necessitates insight into pollinator perception and behavioural responses to the signals which flowers deploy in advertising (Schiestl and Johnson, 2013).

Flowers offer a symphony of synergistic advertising signals to attract pollinators and pollinators use multiple sensory mechanisms whilst foraging to interpret floral displays (Ômura, Honda, and Hayashi, 1999; Goyret and Raguso, 2006; Policha et al., 2016). A complex interplay exists between different floral cues, and the effect and hierarchical nature of advertising signals are often dependant on their context (Kinoshita, Shimada, and Arikawa, 1999; Andersson and Dobson, 2003b; Raguso and Willis, 2005; Balkenius and Dacke, 2010; Cepero, Rosenwald, and Weiss, 2015). The flower constancy demonstrated by pollinators towards certain plants reflects the neural synergy generated through learned floral reward conditioning and the interplay between these adaptive responses and pollinator innate sensory preferences (e.g. colour, shape, scent), which together with morphological traits, impose constraints on foraging (Grant, 1949; Lunau and Maier, 1995; Weiss, 1997). Floral constancy is exhibited by many florivores and manifests as selective foraging in favour of particular flowers, to the disregard of other rewarding flora (Chittka, Thomson, and Waser, 1999). A wide variety of insects including dipterans (Goulson and Wright, 1998), hymenopterans (Grant, 1950; Gross, 1992; Wilson and Stine, 1996; Raine and Chittka, 2005), coleopterans (De Los Mozos Pascual and Domingo, 1991; Mico and Galante, 2005) and lepidopterans (Lewis, 1986; Goulson and Cory, 1993; Goulson, Ollerton, and Sluman, 1997; Goulson, Stout, and Hawson, 1997; Arikawa and Kinoshita, 2000) have been reported to display flower constancy, although some Diptera (Ellis and Johnson, 2012) and Lepidoptera (Pohl, Van Wyk, and Campbell, 2011) do not show marked floral constancy. Because a pollinator's flower constancy governs the quality and quantity of intraspecific pollen export and receipt and has direct consequences for plant fitness, advertising signals may undergo selection pressure if they enhance floral constancy (Schiestl and Johnson, 2013).

Sensory perception by floral visitors varies widely, even within families (Lunau and Maier, 1995) and there exists an equally catholic gamut of learned and innate behavioural responses to the adverting cues offered by flowers (Weiss, 2001). Flower colour is one of the most pervasive of all the adverting cues, playing a key role in pollinator attraction (Menzel and Shmida, 1993; Bradshaw and Schemske, 2003) and is important in maintaining pollinator constancy amongst different floral forms (Waser, 1986; Hill, Wells, and Wells, 1997; Keasar et al., 1997). Innate floral colour preferences have been documented in a wide variety of insects. Diptera, Hymenoptera (bees and bumblebees) and Lepidoptera (butterflies and hawkmoths) are typically attracted to blue (Lunau and Maier, 1995), but some butterfly species also show preferences for longer wavelengths including yellow, orange or red (Ilse, 1928; Crane, 1955; Ilse and Vaidya, 1956; Swihart and Swihart, 1970; Scherer and Kolb, 1987; Weiss, 1997; Kinoshita, Shimada, and Arikawa, 1999; Blackiston, Briscoe, and Weiss, 2011; Hirota et al., 2019). Flower colour can effect ethological isolation (Bradshaw and Schemske, 2003), insomuch as floral morphology influences mechanical isolation (Grant, 1949; Fulton and Hodges, 1999). Pollinators use the overall colour of flowers as well as contrasts in floral colour as cues for foraging (Chittka and Raine, 2006). Simple contrasting colour patterns act as nectar guides, direct pollinators to hidden floral rewards (Sprengel, 1793; Johnson and Dafni, 1998; Dinkel and Lunau, 2001; Lunau et al., 2006; Hansen, Van der Niet, and Johnson, 2012; Goodale et al., 2014), and are widespread throughout the angiosperms (Kugler, 1943; Daumer, 1958; Kugler, 1963; Kevan, 1983; Penny, 1983; Chittka et al., 1994; Weiss, 1995a). Nectar guides promote pollen transfer efficiency (Casper and La Pine, 1984) and at close range perception, insects show attraction bias towards these patterns (Waser and Price, 1983; Waser and Price, 1985; Dafni and Giurfa, 1999). Nectar guides can increase both pollinator foraging efficiency (Waser and Price, 1983; Waser and Price, 1985; Hansen, Van der Niet, and Johnson, 2012) and plant reproductive success (Leonard and Papaj, 2011; Zhang et al., 2017).

A key aspect of angiosperm evolutionary diversification is floral shape variation. Floral shape is typically representative of adaptive mechanical fitting, linking floral architecture with pollinator morphology (Darwin, 1862; Grant and Grant, 1965; Stebbins, 1970) and often promotes mechanical isolation (Grant, 1949). Floral shape has been attributed to pollinator-mediated selection (Grant, 1949) and is a central premise in the pollination syndrome paradigm (Faegri and van der Pijl, 1979). Floral shape divergence between closely related plant species has been shown to produce pollinator shifts (Hodges and Arnold, 1994a; Armbruster, 1996; Fulton and Hodges, 1999; Schemske and Bradshaw, 1999). Like shape, studies have documented changes in pollinator visitation and behaviour in response to modification in floral orientation (Fulton and Hodges, 1999; Tadey and Aizen, 2001; Fenster, Armbruster, and Dudash, 2009; Wang et al., 2014; Campbell, Jürgens, and Johnson, 2016). Although research is sparse, some compelling evidence reports the reduction in visitation to experimental re-orientation of flowers to a pendulous position (Fulton and Hodges, 1999; Giurfa, Dafni, and Neal, 1999; Ushimaru and Hyodo, 2005; Ushimaru, Kawase, and Imamura, 2006). Compared to other floral traits, little is known about the role of orientation in floral isolation, but research is emerging which suggests that floral orientation can play an important role in ethological isolation, acting as a strong floral signal (Fulton and Hodges, 1999; Campbell, Jürgens, and Johnson, 2016).

Advertising cues to pollinators often consist of combinations of visual and odour signals (Raguso, 2004; Dobson, 2006; Leonard and Masek, 2014), and research examining both of these floral traits is increasingly being undertaken (Ômura, Honda, and Hayashi, 1999; Raguso and Willis, 2002; Ômura and Honda, 2005; Raguso and Willis, 2005; Policha et al., 2016). Floral scent plays an important role in long-distance pollinator attraction (Metcalf and Metcalf, 1992; Raguso, 2008) and may endorse pollinator effectiveness and reliability (Dornhaus and Chittka, 1999). Spatial odour patterns may also function as fragranced nectar guides, assisting pollinator orientation (Bergström, Dobson, and Groth, 1995; Lawson, Whitney, and Rands, 2017). Floral scent can increase pollinator specificity (Groth, Bergstrom, and Pellmyr, 1987; Whitten and Williams, 1992; Mant, Peakall, and Schiestl, 2005) and may be correlated with adaptations for food, mate and brood-site location that are found in certain groups of pollinators (Jurgens, Witt, and Gottsberger, 2003; Huber et al., 2005). Floral scent has been shown to shift innate colour preferences from blue to red in female *Papilio xuthus* butterflies (Yoshida et al., 2015).

Lepidoptera show a high degree of diversity in their sensory capacities (for review see Kinoshita, Stewart, and Ômura, 2017). Butterfly vision encompasses some of the most extensive visual systems, with perception ranging from ultraviolet to red (Silberglied, 1984; Koshitaka et al., 2008). Colour has been shown to be a primary foraging cue in a number of butterflies (Weiss, 1995b, 1997; Kinoshita, Shimada, and Arikawa, 1999; Hirota et al., 2012; Cepero, Rosenwald, and Weiss, 2015). Whereas the majority of insects lack receptors for red colours, swallowtail butterflies have red receptors and are able to perceive colours around this wavelength (Briscoe and Chittka, 2001). Butterflies are able to differentiate between contrasting brightness as well as contrasting colours and have true colour vision (Blackiston, Briscoe, and Weiss, 2011; Kinoshita and Arikawa, 2014). Butterflies readily associate food rewards with colour (Swihart and Swihart, 1970; Swihart, 1971; Lewis and Lipani, 1990; Goulson and Cory, 1993; Weiss, 1995b) and learned reward conditioning can result in a rapid shift in innate colour preferences (Ilse, 1928; Swihart and Swihart, 1970; Goulson and Cory, 1993; Weiss, 1997; Kinoshita, Shimada, and Arikawa, 1999; Weiss and Papaj, 2003; Kandori et al., 2009; Kandori and Yamaki, 2012). Butterflies can also associate food rewards with shape (Cepero, Rosenwald, and Weiss, 2015). Flower shape has been suggested to play a role as a facilitative cue which improves the accuracy of foraging bouts in the monarch butterfly (Cepero, Rosenwald, and Weiss, 2015) and corolla curvature has been shown to increase the discovery of nectar by hawkmoths, which also hover while feeding (Campos, Bradshaw, and Daniel, 2015).

Clivia Lindl. (Amaryllidaceae) is a small genus of long-lived evergreen forest understory species endemic to southern Africa (Conrad et al., 2006). A shift from bird to butterfly pollination is likely to have occurred during the diversification of *Clivia* (Kiepiel and Johnson, 2014). *Clivia miniata* Lindl. Bosse occupies a derived position and is the only member of the genus with upright weakly-scented trumpet-shaped flowers and is pollinated primarily by swallowtail butterflies, while the other four species have narrow unscented pendulous flowers and are all sunbird-pollinated (Kiepiel and Johnson, 2014). The upright floral orientation and trumpet-shape of *C. miniata* flowers provide a landing platform, and facilitate transfer of pollen on butterfly's wings as they access nectar (Kiepiel and Johnson, 2014). In addition, flowers of *Clivia miniata* are unique in the genus in having distinct central target-shaped nectar guides and emission of scent. Flowers of *C. miniata* are usually orange with a central yellow target, but there is a rare morph (var. *citrina*) with all yellow flowers. *Clivia* represents a unique opportunity to examine the function of floral traits important for butterfly pollination in a clade where the ancestor can be reliably inferred to have typical pendant tubular bird-pollinated flowers.

We aimed to identify traits responsible for attraction of butterflies to flowers of *C. miniata*. Based on the distribution of traits across species in the genus, we hypothesized (1) that butterflies will favour orange flowers with a central yellow target pattern over other colours and patterns, (2) that butterflies will be more attracted to, and more likely to settle on, flowers which face upwards, (3) that butterflies will show electrophysiological responses to scent compounds emitted by *C. miniata* and prefer scented over unscented flowers.

METHODS

STUDY SITES – Behavioural experiments were performed in the natural forest habitats of *Clivia miniata* during the Austral spring (flowering season August to October) from 2010-2017, at two sites in KwaZulu-Natal Province, South Africa. One site was inland (approximately 90 km from the coast), located in the mistbelt forests of the Karkloof Midlands (Mbona Private Nature Reserve, 29°17'S; 30°21'E, ca, 1300 m a.s.l., hereafter referred to as MPNR). The other was a coastal site, situated in coastal scarp forest (Umtamvuna Nature Reserve, 31°00' S, 30°09' E, ca. 150 m a.s.l., hereafter referred to as UNR). These two sites are located approximately 185 km apart. *Clivia miniata* is listed as vulnerable in the Red List of South African Plants (Raimondo et al., 2009), therefore detailed plant localities beyond the area in which they are found are omitted. Details of plant localities are provided in the Bews Herbarium (NU), University of KwaZulu-Natal, Pietermaritzburg (NU0038189; NU0038190). Bioassays were conducted only during the period when *C. miniata* was in flower and experimental arrays were placed in the vicinity of flowering plants.

SPECTRAL ASSESSMENT AND MODEL DESIGN – Floral spectral reflectance measurements of the corolla of *C. miniata* were taken from flowers at the MPNR and UNR sites. *Clivia miniata* var. *citrina* floral spectra were taken from flowers originating from nursery raised plants (Cycad Centre, KwaZulu-Natal, RSA), as plants are extremely rare in the wild. Additional floral spectral reflectance measurements from the co-occurring pendulous flowered congener *C. gardenii* Hook. were taken from flowers at the UNR site, a site relatively close in proximity to the UNR site (Pondoland, 30°50' S, 30°00' E, ca. 350 m a.s.l., hereafter referred to as Pondoland) and an inland site (Gibba Gorge, 29°48' S, 30°46' E, ca. 550 m a.s.l., hereafter referred to as GG). We also sampled floral spectra of other forest and forest margin species visited by butterflies in the same community to assess variation in the spectral reflectance of butterfly-visited flowers. Spectral reflectance (300 -700 nm) was measured using an Ocean Optics S2000 spectrophotometer (Ocean Optics, FL, USA) coupled with a fiber optic reflection probe (QR-400-7-UV-VIS; 400 µm, Johnson and Andersson, 2002). Spectra were graphed using Endler colour space segment classification (Endler, 1990).

Paper model flowers were constructed to match the shape and spectral reflectance of *C. miniata* flowers. Artificial flowers were also constructed to match the flower shape of bird-pollinated *C. gardenii* and the reflectance spectra of other butterfly-visited species. Bright paper board (Star Paper Products, A4 project boards, Brilliant Bright Board, 160 gsm) were used for blue, green, orange and red colours, whilst paper (Marlin, A4 Pastel Paper Pad, 80 gsm) was used for pink and yellow (see Figure 2A for paper spectral reflectance). Yellow and pink model paper had a close similarity in their respective ultraviolet components (Figure 2A). When plotted in Endler's segment classification (Endler, 1990), yellow and pink model paper appeared very close to one another due to this similarity in ultraviolet wavelength (Figure 2B), although the two colours were quite distinct in other regions of the spectrum (Figure 2A). *Clivia miniata* models (e.g. Figure 1C) consisted of simple cones, scaled to previously recorded perianth dimensions for length (approximately 74 mm) and entrance width (approximately 73 mm, Kiepiel and Johnson, 2014). *Clivia miniata* models were manufactured from 150 mm circular disks and models with centres of differing colours consisted of another 90 mm disk overlaid onto the first so that the central colour terminated 30 mm from the cones diameter. A single cut was made along the radius of the disk and the paper cone glued in place using non-toxic, solvent free glue (Pritt, Henkel AG & Co.). *Clivia miniata* reduced size models were simply a 3 x reduction in size. *Clivia gardenii* models were manufactured similarly, with a perianth length of approximately 40 mm and an entrance width of approximately 10 mm (Kiepiel and Johnson, 2014).

Artificial pedicels were constructed from twisted green plastic-coasted wire (Garden Jem, PVC coated garden wire, 2 mm) and were attached to 'peduncles' manufactured from 1 m long wooden dowel rods (8 mm diameter), which were painted green (Spectra Spray, lead free aerosol spray paint, brilliant green). Four 'pedicels' were placed onto each 'peduncle', the wire from which was inserted into the base of the model paper flower (ensuring that the 'pedicel' was not protruding into the flower) to create each artificial inflorescence (Figure 1C). Model flowers were equidistantly spaced around the peduncle and positioned at an angle of 45 degrees to the vertical, matching *C. miniata*, or bent downwards (approximately 10 degrees from vertical) to match pendulous *C. gardenii* flowers (Kiepiel and Johnson, 2014). The 'peduncle' of the artificial inflorescence was positioned at a similar height (c. 80 cm) to that of the surrounding *C. miniata* inflorescences.

MODEL FLOWER ARRAYS – Choice experiments began in the morning, prior to the commencement of butterfly activity (06h00), and trials were terminated when butterfly activity had ceased (c. 16h00). Butterflies typically became active around 08h00 and were not on the wing during overcast, rainy or cold weather, with activity ceasing entirely below 22ºC. Short flowering seasons coupled with inclement weather necessitated numerous years of experimentation. Each bioassay consisted of a choice between two or more model inflorescences, which were placed approximately 100 cm apart. The positions of model inflorescences were randomised every 10 minutes in order to avoid any potential position effects. The time of day of each visit, species and sex of butterfly was recorded. The following behaviours were recorded: approaches (within 15 cm of model), when real interest was observed involving clear intent and definitive lines of inquisition, (e.g. Figure 1A); brushes, when the butterflies did not settle and brushed the models with wings or legs (e.g. Figure 1D), and alights when butterflies landed on the models (e.g. Figure 1B & E). Visit duration, probing behaviour and probing time were also recorded.

To test butterfly response to various colours representative of butterfly-visited flowers in the community, we used an array of *C. miniata*-shaped models consisting of five plain (unpatterned) colour choices; pink, blue, yellow, orange and red (see supplementary Table S1 for all site details and experimental duration).

To test the response of butterflies to colour patterns, we used a model array comprising of six *C. miniata*-shaped choices (three plain colours and three with targets); orange, red, yellow, orange with yellow target, red with yellow target and yellow with red target models. We used orange with yellow target models as they closely mimic *C. miniata* colouration, and the other combinations as being representative of the range of possible combinations based on perianth colours found in *Clivia* (see Figure 1B).

To simplify and further test the effect of contrasting coloured targets and simple nectar guides in butterfly attraction, we used a model array consisting of only two *C. miniata*-shaped choices; plain orange versus orange with yellow target models.

To test the effects of flower size on butterfly attraction, we used a model array consisting of two *C. miniata*-shaped choices; plain orange versus a reduced-size (one-third scale) plain orange models.

To test the combined effects of size, shape and orientation, and whether butterflies are attracted to pendulous flowered *Clivia*, we used a model array consisting of two choices, representing two *Clivia* species; plain orange *C. miniata*-shaped models *versus* plain orange *C. gardenii*-shaped models.

To test the role of scent in butterfly attraction we used a model array consisting of two *C. miniata*-shaped choices, comprising of orange with yellow target models; one with an artificial *C. miniata* scent blend (housed in a small vial in the centre of the 'inflorescence') and one with a paraffin control (1000 microliters, Figure 1C). This scent blend was mixture of equal proportions of Benzyl Alcohol (Sigma-Aldrich), Benzaldehyde (Sigma-Aldrich) and Benzyl benzoate (Sigma-Aldrich) - 5 microliters of each compound into 985 microliters unscented paraffin liquid. These three compounds were chosen for their ubiquitous presence in *C. miniata* scent (Kiepiel and Johnson, 2014). Scent vials were shaken every 10 minutes and replaced every hour, and scented models were kept separate to unscented models to prevent scent contamination.

ARRAYS WITH REAL FLOWERS– To assess whether butterflies prefer the common orange morph of *C. miniata* over the rarer yellow morph (var. *citrina*), we compared rates of butterfly visitation to the two morphs using a paired design. *Clivia miniata* and *C. miniata* var. *citrina* plants were bought in bud from a nursery (Cycad Centre, KwaZulu-Natal, South Africa) and placed into a 2 x 2 x 2 m pollinator exclusion cage, where they were emasculated prior to anthesis to prevent genetic contamination with wild plants. Buds were allowed to open naturally and when eight flowers on an inflorescence were open (only eight flowers per inflorescence were used in order to standardise the assay), the peduncles were cut and taken into the forest for choice tests. Peduncles were then fixed to green painted wooden dowels. All real flower arrays were randomised every 10 minutes to avoid position effects. Bioassays consisted of a single *Clivia miniata* var. *miniata* inflorescence versus a single *C. miniata* var. *citrina* inflorescence with inflorescences replaced with fresh ones daily (layout and execution as with models, see supplementary Table S1 for details).

To test the effect of floral orientation on butterfly visitation, two choices were presented; *C. miniata* flowers in their usual upright orientation versus *C. miniata* flowers manipulated into a pendulous orientation (Figure 1D). Flowers were bagged in the field using pollination bags and allowed to open naturally, picked at anthesis and tied to artificial pedicels in either upright or pendulous orientation (four per treatment). Flowers were replaced with fresh ones daily.

To test the effect of scent supplementation on butterfly visitation to live flowers, inflorescences were bagged as above, harvested, and scent and control paraffin vials (methods identical to model flower experiment) were hung from the pedicels of the inflorescence. Inflorescences were harvested for use once eight flowers were open and replaced with fresh ones daily.

ELECTROANTENNOGRAPHIC DETECTION – To examine butterfly antennal responses to volatile compounds found in *C. miniata* floral scent, electroantennographic detection (EAD) was performed on swallowtail butterflies (*P. dardanus cena, P. echerioides echerioides, P. ophidicephalus, P. nireus lyaeus, P. demodocus*) and the common forest white butterfly (*Belenois zochalia zochalia*) caught at MPNR. An equal number of male and female butterflies were caught, twelve of each species in October 2016 and a further 12 in October 2017. We opted to use an artificial scent blend of some of the floral volatiles emitted by *C. miniata* (Kiepiel and Johnson, 2014) in order to standardize emission rates and eliminate variability through discrepancies in headspace sampling. The artificial blend consisted of one microliter each of limonene, benzaldehyde, linalool, benzyl acetate, benzyl alcohol and benzyl benzoate (all Sigma-Aldrich), which was added to 1994 microliters of acetone. Following the methods of Shuttleworth *et al.* 2017, this blend was injected into a clean scent cartridge and placed into a Bruker 1079 injector fitted with a ChromatoProbe thermal desorption device attached to a Bruker 450 gas chromatograph (Bruker BR-SWAX column, 0.25 mm x 30 m internal diameter, 0.25 µm film thickness) coupled with a Bruker 300 quadrupole mass spectrometer (70 eV in electron impact ionization mode, detector response set by EDR), with EAD responses recorded simultaneously with outputs of the mass spectrometer (GC-MS-EAD). An Ockenfels Syntech GmbH (Germany) EAD device, using GCEAD v4.4 software (Syntech) was used to visualize antennal responses. Prior to antennae excision, each individual was administered with carbon dioxide for sedation. Both antennae were used for EAD.

Antennae were excised as close to the head as possible and mounted using glass pipettes (held with a MP-15 micromanipulator) containing a ¼ strength RINGER solution (Merck, Germany), the distal end of which were connected using silver wires to a EAG Combi Probe attached to a 2-channel USB Acquisition Controller (IDAC-2), according to Shuttleworth *et al.* 2017. A CS55 Stimulus Controller was used to provide a flow of humidified, filtered air (rate, approximately 41 min^{-1}) directly from the tip of the column, which carried GC volatile effluent to the prepared antennae at a distance of approximately 10 cm (Shuttleworth, Johnson, and Jurgens, 2017).

STATISTICAL ANALYSIS – Statistical analyses used generalized linear models implemented in SPSS Version 25 (IBM Corp.). Generalized estimating equations (GEEs) were used to analyse the number of butterfly approaches, brushes and landings per hour. Each hour-long observation period was used as a subject in the GEE to control for lack of independence among the observations in that period, which could be due to factors such as time of day, weather conditions etc. Counts of butterfly behavioural events were modelled with a negative binomial distribution with a log link function, while the proportions of butterflies choosing a particular model or real flowers was modelled using a binomial distribution with a logit link function. First choice proportions were considered significant if 95% confidence intervals did not overlap the 0.5 proportion value of equal choice among two options. GEE models incorporated an exchangeable correlation matrix and significance was tested using Wald Statistics, as Score statistics are overly conservative with regard to small sample sizes (Guo et al., 2005). In some cases, where models failed to run due to lack of variance (e.g. where butterfly visitation to an artificial or live flower array was zero) a single value was substituted, which makes the test more conservative (Zuur et al., 2009). The significance between means was evaluated using the sequential-Šidák method in order to adjust for multiple comparisons. For graphical presentation of marginal means in the original scale measurement, log or logit data from the linear scale were back-transformed to obtain asymmetrical standard errors.

RESULTS

SPECTRAL ASSESSMENT –*Clivia miniata* and *C. gardenii* showed significant floral colour variation both within and between populations when visualized in Endler segment classification (Endler, 1990, Figure 2B). Colouration of *C. miniata* flowers ranged from red to orange and yellow hues. *Clivia miniata* flower colours showed a parallel to those of *C. gardenii*, although flower colour in *C. gardenii* was far more variable (Figure 2B). *Clivia miniata* var. *citrina* showed a distinct colour grouping separate to that of *C. miniata* and closer to that of *C. gardenii* at the UNR (Figure 2B).

MODEL FLOWER ARRAYS – Models in the five-colour choice array were visited almost exclusively by *Papilio dardanus cena* (Figure 3), with the exception of two *P. nireus lyaeus* individuals, which were omitted from the analyses (see supplementary Table S1 for all statistical details). *Papilio dardanus cena* showed significant discrimination amongst colours with pink approached least and orange the most (Figure 3A). *Papilio dardanus cena* displayed no significant preference in the number of brushes or alights per hour between colours, but the sample sizes of butterflies that exhibited these behaviours were low (Figure 3B and C).

Models in the colour pattern choice array were visited by five species of butterflies (*Nepheronia argia, Papilio euphranor, P. dardanus cena, P. ophidicephalus, P. echerioides echerioides*), the data for which were combined in analyses (Figure 4). Model colour significantly influenced approaches and alights, but had no impact on the number of brush visits (Figure 4). Targets alone did not significantly affect butterfly visitation (Figure 4A-C), but there was a significant statistical interaction between colour and the presence of targets in the number of approaches per hour (Figure 4A). With regards to approaches, orange, red, and orange with yellow target models were significantly favoured over plain yellow models (Figure 4A). No significant difference was found between the hourly number of approaches to orange, red, and any of the models with targets, but mean values were low for these behaviours (Figure 4A, supplementary Table S1). The number of hourly alights on the yellow models with a red target were significantly lower than those on the other models (Figure 4C).

The orange target no-target pair (plain orange versus orange with a yellow target) was visited entirely by *P. dardanus cena* (Figure 5), with the exception of two *P. nireus lyaeus* individuals, which were omitted from the analyses. Target models were significantly preferred over plain models in terms of visits per hour (Figure 5) and in terms of binomial proportion (Supplementary Table S1, first choice analysis). *Papilio dardanus cena* approached and brushed the target models a significantly greater number of times per hour than they did those of the no-target models (Figure 5A & B). There were no alights in the models in this experiment.

Regular versus one-third size models were visited exclusively by *P. ophidicephalus* (Figure 6). *Papilio ophidicephalus* significantly preferred larger models in terms of approaches per hour (Figure 6), which was also reflected in the first choice proportion (Supplementary Table S1, first choice analysis). No significant trends were found in terms of the number of brush or alight visits to these models, but these sample sizes were very low and a meaningful comparison could not be made (Figure 6B & C).

Four swallowtails and a pierid visited the *C. miniata* versus *C. gardenii* model array (Figure 7). We found a significant difference in the number of approaches per hour for *P. ophidicephalus,* which strongly favoured *C. miniata* shaped models (Figure 7A), however no other significant trends were found, which may reflect the small number of butterflies involved in brush and alighting behaviours (Figure 7B & C).

Unscented target versus scented target arrays were approached by five butterfly species in MPNR (Figure 8A, C & E) and three in the array at UNR (Figure 8B, D, F). Over 65% of butterflies at MPNR and almost 60% of butterflies at UNR approached scented models (Supplementary Table S1, first choice analysis). A significant overall difference in choice was found in the number of approaches and brushes per hour only at the MPNR site (Figure 8A & C), but pair-wise comparisons did not show any significant differences in approaches for any specific species. No significant interaction was found between butterfly species and the scent treatment for any behaviour (Figure 8), but brush visits made by *P. nireus lyaeus* to scent-supplemented models were significantly more frequent than those to unscented models (Figure 8C).

ARRAYS WITH REAL FLOWERS – Arrays involving *C. miniata* var. *miniata* versus *C. miniata* var. *citrina* at UNR were visited exclusively by *P. dardanus cena* in the 2013 season and in the 2014 season by *P. dardanus cena* and two additional species (Figure 9). In the 2013 season, 68% of butterflies first approached var. *miniata*, whilst in the 2014 season, approximately 77% of butterflies approached var. *miniata* flowers (Supplementary Table S1, first choice analysis). In both the 2013 and 2014 season, var. *miniata* flowers were approached, brushed and alighted on a significantly greater number of times per hour by *P. dardanus cena* than were var. *citrina* (Figure 9). *Belenois zochalia zochalia* also alighted significantly more on var. *miniata* inflorescences than those of the yellow morph (Figure 9F). A significant difference in preference was found between butterfly species, but there was no significant interaction between species and choice between the two colour morphs in the 2014 season (Figure 9B, D & F).

Papilio dardanus cena and *P. nireus lyaeus* were the only visitors to the pendulous versus upright arrays of real *C. miniata* flowers (Figure 10). There was no overall difference in first choice amongst the two orientations (Supplementary Table S1, first choice analysis). A significant overall difference in choice was found in the number of brushes per hour, but pair-wise comparisons showed no significant differences in brushes for either *P. dardanus cena* or *P. nireus lyaeus* (Figure 10B). No significant difference was found in the number of approaches or brush visits between the orientations (Figure. 10A), however *P. dardanus cena* individuals alighted significantly more often on the upright oriented flowers (Figure 10C).

Belenois zochalia zochalia, *P. dardanus cena* and *P. nireus lyaeus* visited the unscented control versus scent supplemented real flower arrays (Figure 11). A significantly higher proportion of butterflies chose the scent-supplemented inflorescences (Supplementary Table S1, first choice analysis). Individuals of *P. dardanus cena* approached scent supplemented flowers significantly more frequently than they did the control flowers. No significant overall difference in choice was found in the number of brushes or alights per hour (Figure 11B & C), but pair-wise comparisons showed a significant difference in the brushes for *P. dardanus cena* (Figure 11B). There were no significant interactions between choice and butterfly species (Figure 11).

ELECTROANTENNOGRAPHIC DETECTION – Antennal responses were obtained from *P. dardanus cena* and *P. demodocus* (Figure 12). Antennae of male *Papilio dardanus cena* butterflies showed a response to acetone and benzaldehyde (Figure 12A), whilst females showed weak responses to limonene, benzaldehyde as well as benzyl alcohol and benzyl benzoate (Figure 12B). Antennal reactions from *Papilio demodocus* were only obtained by males, which showed weak responses to benzaldehyde, benzyl alcohol and benzyl benzoate and less clearly, a response to benzyl acetate (Figure 12 C). Overall, very few antennal responses were observed in *P. dardanus cena* and *P. demodocus* and we were unable to obtain responses from the swallowtails *P. ophidicephalus*, *P. nireus lyaeus* or *P. echerioides echerioides. Belenois zochalia zochalia* also did not show any meaningful EAD responses.

DISCUSSION

Results from this study demonstrate that colour is used as a primary advertising cue by the assemblage of butterflies that visit *C. miniata* in South African forests and thus plays a fundamental signalling role in that plant species. This is consistent with studies of other butterflies (Ilse, 1928; Goulson and Cory, 1993; Weiss, 1995b; Kinoshita, Shimada, and Arikawa, 1999; Ômura and Honda, 2005). Butterflies showed clear behavioural responses involving directional flight pattern adjustment towards the target stimulus from distances of up to 15 metres. *Clivia miniata* flowers attract numerous butterfly species (Kiepiel and Johnson, 2014) and floral colouration is likely a product of pollinator-mediated selection driven by this diverse lepidopteran assemblage. Because the suite of butterflies in the forest community fluctuates on a seasonal and geographical scale (Kiepiel and Johnson, 2014; Figure 8 & 9), floral colouration in *C. miniata* would be expected to accommodate the preferences of several pollinating butterfly species to make full use of the spatiotemporal variation in this assemblage. Butterfly colour vision is amongst the most extensive of all animal groups and chromatic perception can range from UV to red (Silberglied, 1984; Briscoe and Chittka, 2001; Koshitaka et al., 2008). Swallowtails such as the Japanese *Paplilio xuthus* have true colour vision (Kinoshita, Shimada, and Arikawa, 1999), with four colour opponencies (red, green, blue and UV), providing a tetrachromatic visual system that has one of best known levels of wavelength discrimination (Koshitaka et al., 2008).

This highly developed visual acuity of butterflies presupposes the importance of optical perception in foraging and our experiments with the assemblage of butterflies that visit *C. miniata* indicated that even simple coloured disks (pink, blue, yellow, orange and red) were able to elicit approach visits (I Kiepiel, unpublished data). However these models seldom elicited brushes and alights. Only 6% of butterflies that approached the five-colour array alighted on the models (Figure 3, Supplementary Table S1), compared to 49% over two seasons (2013 and 2014) in the array with real flowers (Figure 9, Supplementary Table S1). This suggests that while colour is a key attractant, at very close-range distances such as brush visits, the visual details of flowers or scent is required to elicit landing behaviour. Research into innate colour preferences of *P. xuthus*, indicates that naïve (three day starved) butterflies readily alight on simple unrewarding coloured disks, frequently landing on the periphery, leading the authors to suggest that edge recognition plays an important role in butterfly settling behaviour (Kinoshita, Shimada, and Arikawa, 1999). It is likely that a combination of learning and satiation in our field experiments with butterflies did not give the same result as flight cage data with naïve starved animals with respect to alights on unrewarding colour stimuli. In our study, we also found that alighting butterflies frequently made contact with the lip of the corolla in real flower arrays and similarly, in artificial arrays, used the edge of the model for coordinating brush visits or a suitable settling position. *Papilio xuthus* has been shown to use the intensity contrast between target and background when landing, relying on the edge of the target for locating a suitable landing position (Koshitaka, Arikawa, and Kinoshita, 2011). In the cabbage white butterfly, *Pieris rapae*, similar results from electroantennography (EAG) and proboscis extension reflex (PER) experiments suggest that floral volatiles may be utilized in flower location and recognition at close-range distances rather than being a long-range olfactory cues (Ômura, Honda, and Hayashi, 1999). When butterflies did alight on models, visit duration was typically no more than a few seconds and probing was seldom apparent. The scarcity of butterfly alights to model flowers points towards chemoreception and butterflies also appear to use tactile perception through brush visits as inspections in order to evaluate the flowers. This is probably because of the role of tactile contact chemoreception provided by chemosensilla responses of butterfly tarsi in feeding and oviposition (Takeda, 1961; Fox, 1966).

The yellow flowered *C. miniata* var. *citrina* is very rarely found in the wild and the five-colour array tests at UNR revealed that orange was strongly favoured by *P. dardanus cena* in approaches over yellow models (Figure 3A). *Papilio dardanus cena* is a key pollinator of *C. miniata* – in a previous study at the UNR site, this butterfly was responsible for approximately 84% of visits to *C. miniata* flowers (Kiepiel and Johnson, 2014). Swallowtails show variable innate colour preferences between species; some such as *Papilio machaon* (Ilse, 1928) and *Papilio demoleus* (Ilse and Vaidya, 1956) prefer blue and purple, others such as *Papilio xuthus* favour yellow and red (Kinoshita, Shimada, and Arikawa, 1999), whilst *Papilio troilus* prefers blue as a primary and orange as a secondary choice (Swihart, 1970). Butterfly pollinated plants display an equally diverse assortment of flower colours, ranging from red, orange, yellow, red, purple, mauve and pink (Kevan and Baker, 1983). We could not determine whether the colour preference for orange hues found in this study represents innate responses or is learned behaviour from experiential feeding on the orange *C. miniata* flowers, as many butterflies are able to equate nectar rewards with colour (Ilse, 1928; Ilse and Vaidya, 1956; Goulson and Cory, 1993; Weiss, 1995b, 1997; Kinoshita, Shimada, and Arikawa, 1999). A growing body of literature indicates that butterflies are able to associate colours with nectar rewards (Goulson and Cory, 1993; Weiss, 1995b; Kandori and Ohsaki, 1996; Weiss, 1997; Kinoshita, Shimada, and Arikawa, 1999; Weiss and Papaj, 2003; Blackiston, Briscoe, and Weiss, 2011). Although our result may simply point towards butterfly learning, whereby the butterflies have associated orange with nectar rewards, a number of species show innate preferences for orange and red wavelengths (Ilse, 1928; Crane, 1955; Ilse and Vaidya, 1956; Swihart and Swihart, 1970; Scherer and Kolb, 1987; Kandori et al., 2009; Blackiston, Briscoe, and Weiss, 2011; Kandori and Yamaki, 2012), and butterfly-pollinated flowers of red wavelengths are not uncommon in South Africa (Johnson, 1994; Johnson and Bond, 1994; Goldblatt and Manning, 2002). Equally, butterflies display floral constancy (Goulson and Cory, 1993; Kandori and Ohsaki, 1996) and a number of species feed only on certain flowers, disregarding the flowers of a range of flora (Lewis, 1989; Weiss, 1995b; Kandori and Ohsaki, 1996; Goulson, Ollerton, and Sluman, 1997). Field caught *P. dardanus cena*, *P. nireus lyaeus*, *P. ophidicephalus* and *P. echerioides echerioides* will readily feed on blue (as well as orange) model flowers with artificial sucrose rewards (I, Kiepiel, unpublished data), and were observed to regularly feed on flowers of blue-hued marginal forest species, such as the ribbon bush *Hypoestes aristata* Soland. Ex Roem & Schult., the blue flowered cape leadwort *Plumbago auriculata* Lam., as well as grassland species such the blue lily *Agapanthus praecox* Willd. (I, Kiepiel, personal observation). If blue and orange are not innate preferences in these butterflies, they are likely colours for which the butterflies can rapidly develop an affinity towards.

Like the five-colour model experiment, results from the real flower array showed that the orange flowers of *C. miniata* were generally far more attractive to butterflies than the yellow flowers of *C. miniata* var. *citrina* over two consecutive seasons at the UNR site (Figure 9.). Although butterflies were attracted to the yellow flowers of *C. miniata* var. *citrina* inflorescences (Figure 1E, Figure 9), first choice analysis indicated that approximately only 32% of butterflies in the 2013 season and 23% in the 2014 season approached the yellow variety of *C. miniata* (Supplementary Table S1). This helps to explain why the yellow form of *C. miniata* is so rare in the wild. Results of the colour pattern array of this study, showed a similar trend to that of the five-colour array (Figure 3) and the real flower array (Figure 9), where orange was strongly favoured over yellow (Figure 4A). The striking parallel in floral morphology between *C. miniata* and the butterfly-pollinated *Hemerocallis fulva* L. (Hirota et al., 2019), and *C. miniata* var. *citrina* and the hawkmoth pollinated *H. citrina* Baroni (Hirota et al., 2019) is intriguing. Although moth pollination in *C. miniata* has been ruled out (Kiepiel and Johnson, 2014), field based observation did not involve night observations of reciprocal transplants of the yellow flowered *C. miniata* var. *citrina*. The scarcity of wild *C. miniata* var. *citrina* implies that yellow colouration has not been selected for by butterflies or moths, however the possibility of a pollinator shift from butterfly to hawkmoth pollination in *C. miniata* var. *citrina* cannot be entirely dismissed.

The presence of contrasting coloured targets did not influence butterfly visitation in the colour pattern array (Figure 4A-C). Some Pierids such as the cabbage white, *Pieris rapae* are stimulated by nectar guides which promote proboscis extension in the foraging butterflies (Kandori and Ohsaki, 1996, 1998) and it may be that with the butterfly assemblage visiting *C. miniata*, certain species react more strongly to this advertising stimulus than others. A recent examination of UV bullseye patterns in *Hemerocallis* L. indicates that preferences of the swallowtail *P. xuthus* were influenced by the peripheral and central colour of flowers and not by contrasts in bullseye patterning (Hirota et al., 2019). This research suggests that although swallowtails recognise contrasting bullseye targets, varying contrast intensity had no significant influence on visitation (Hirota et al., 2019). Our results did not support an increased attraction bias to models with contrasting targets in the colour pattern array when visited by five butterfly species (Figure 4). Conversely, as opposed to our colour pattern array (Figure 4), a preference was found in *P. dardanus cena* for targeted compared to plain orange models in the orange target no-target paired array in this study (Figure 5). This disparity between the orange target no-target paired array (visited only by *P. dardanus cena,* Figure 5) and that of the colour pattern array (Figure 4), may lie in the particular visual attributes of each species in the suite of visiting butterflies present in each experiment, as the colour pattern array was visited by five species - one pierid (*Nepheronia argia*) and four swallowtails (*P. euphranor, P. dardanus cena, P. ophidicephalus, P. echerioides echerioides*). In *Pieris rapae*, experiments with artificial arrays indicated that both flower constancy and foraging efficiency were heightened by bullseye floral pattering (Kandori and Ohsaki, 1998). Results from the orange target no-target paired array (Figure 5) showed that *P. dardanus cena* has a strong preference for contrasting targeted patterning. This result alludes to dissimilar preferences in contrasting targeted patterns between butterfly species in the assemblage visiting *C. miniata*.

The orange paper we used for our models had a moderate UV component (Figure 2A), but UV reflectance of the yellow model paper was more than three times higher, providing for not only a colour contrast in 400-700 nm wavelengths, but also that of a UV contrast. In the study of *Hemerocallis* L., *P. xuthus* showed preferences for peripheral and central floral colouration rather than bullseye contrast (Hirota et al., 2019). This suggests that the UV contrast (or 400-700 nm contrasts between target and periphery) between orange and yellow model paper in our study was of negligible importance in influencing butterfly visitation and rather, the central and peripheral hues of yellow and orange respectively, (rather than their contrast), play a more substantial role in effecting visitation. Attraction to certain floral cues such as colour may not automatically reflect innate pollinator preferences, but may instead indicate associative conditioning between rewards (e.g. nectar, pollen etc.) and signal stimuli (Menzel, 1979). It is possible therefore that the preference for target patterns was learned through association with local *C. miniata* plants. With the exception of butterflies (Kinoshita, Shimada, and Arikawa, 1999), the lack of red receptors in the majority of insects including moths, bees and flies (Lunau and Maier, 1995), imposes to some degree, a form of chromatic floral isolation on red flowers (Rodriguez-Girones and Santamaria, 2004). *Clivia miniata's* delicate flowers are too fragile for red-wavelength perceptive sunbirds to effectively land upon, and the prevalence of red flowered *C. miniata* over the yellow *C. miniata* var. *citrina*, is suggestive of butterfly-mediated selection.

Results from our arrays with differing model flower sizes, indicated that larger size models attracted significantly greater number of approaches by *P. ophidicephalus* compared to one-third scale models, which generated very little interest (Figure 6). This may illustrate the importance of large flower size as an advertising signal, as bigger floral displays correlate to more visits (Klinkhamer and de Jong, 1990; Ohara and Higashi, 1994; Robertson and Macnair, 1995; Goulson et al., 1998; Vrieling et al., 1999; Mitchell et al., 2004; Grindeland, Sletvold, and Ims, 2005; Makino, Ohashi, and Sakai, 2007). Although rates of pollinator visitation are influenced by floral size (Mulligan and Kevan, 1973; Wilson and Price, 1977), aversion to smaller flowers may also represent rewarded conditioning on the larger *C. miniata* flowers as many swallowtails do visit other small orange flowers such as those of the wild pomegranate *Burchellia bubalina* (L.f.) Sims (I, Kiepiel, personal observation). Overall floral display size may be an important advertising trait in *C. miniata* as plants that are isolated in the forest, far away from the main body of the population also obtain far fewer visits from butterflies and produce fewer seeds when compared to groups of plants that are in close proximity to each other (I, Kiepiel, personal observation). However, a number of reports describe preferences to both larger floral displays and flower sizes in Lepidoptera (Vaughton and Ramsey, 1998; Arroyo et al., 2007; Pohl, Van Wyk, and Campbell, 2011).

Results from the *C. gardenii* versus *C. miniata* choice model array showed that *P. ophidicephalus*, strongly favoured *C. miniata* shaped models, but other species were indifferent (Figure 7). *Papilio ophidicephalus* is the biggest butterfly species in southern Africa and model flowers of *C. gardenii* offered a completely unsuitable orientation for butterflies to alight. Floral size may not be a limitation to butterflies, but orientation imposes a strict limitation to the butterflies, particularly when no landing platform is available. For the most part, the pendulous shape of *C. gardenii* effectively excludes butterflies from visiting the flowers as it offers no purchase for butterflies on which to alight. However, exceptionally rarely, butterflies have been observed visiting pendulous *Clivia* species, alighting on the periphery of the inflorescence and attempting to feed whilst clinging to the lip of the corolla (I. Kiepiel, personal observation). Such rare interactions are hardly typical, but like the *C. gardenii* versus *C. miniata* choice model array (Figure 7), this behaviour is suggestive of the relative strength of floral colour cues over shape or orientation, and highlight the exploratory inquisitiveness of foraging butterflies. The putative shift from bird- to butterfly-pollination in *Clivia*, was most likely underpinned by the modification of floral orientation, with the upturning of flowers, facilitating a shift from highly specialized bird-pollination to a more generalized pollination system likely involving both birds and insects. Spectra of congeners (Figure 2B) indicate striking similarities in floral coloration between the pendulous flowered species and that of *C. miniata*. *Clivia miniata's* orange hues were likely maintained during the putative shift from bird- to butterfly-pollination (Kiepiel and Johnson, 2014) and may have served as an exaptation for butterfly pollination. Experimental manipulation of floral orientation in *Geranium refractum* Edgew. & Hook.f. from a downward- to an upward-facing orientation, has been found to shift pollination from specialization (i.e. bumblebees) to generalization, with an associated reduction in pollen transfer efficiency (Wang et al., 2014). In *Zaluzianskya* F.W.Schmidt, manipulation of floral orientation has been shown to cause a shift in hawkmoth pollination, providing compelling evidence for the role of floral orientation in pollinator shifts and reproductive isolation (Campbell, Jürgens, and Johnson, 2016).

Results from the upright versus pendulous *C. miniata* flower array showed no difference in the number of approaches made by either *P. dardanus cena* or *P. nireus lyaeus* (Figure 10), indicating that at a distance, colour appears to be take priority over orientation in real flowers. Although this contrasts with the results from the experimental array consisting of *C. gardenii* versus *C. miniata* models (Figure 7), *P. ophidicephalus* was not present at the real flower array at the UNR site (Figure 10), and is not common in coastal forests (Kiepiel and Johnson, 2014), which may explain the difference. This also again highlights the differences in preferences of different butterfly species. After approaching, butterflies showed an exceptional curiosity and interest in the pendulous flowers, inspecting the real flowers during brush visits (Figure 10). Although *P. nireus lyaeus* did not alight nearly as many times as *P. dardanus cena*, the complete absence of alights to pendulous orientated *C. miniata* by both species indicate that there would be strong selection again pendant orientation during the shift from bird to butterfly pollination. Studies of floral isolation between *Aquilegia formosa* Fisch. ex DC. and *Aquilegia pubescens* Coville indicate that manipulation of *A. pubescens* flowers into a pendent orientation, reduced hawkmoth visitation by tenfold (Fulton and Hodges, 1999). Here, orientation influenced floral isolation through pollinator behavioural modifications, whilst mechanically, floral isolation was promoted by nectar spur length (Fulton and Hodges, 1999; Hodges, 2002). In *Silene virginica* Willd., floral orientation has been found to act independently of floral symmetry by directing the movement of pollinators, which has important fitness consequences for plants (Fenster, Armbruster, and Dudash, 2009). Recent research has demonstrated that floral orientation is critical for *Manduca sexta* pollination of *Nicotiana attenuata* Steud. (Yon et al., 2017; Haverkamp et al., 2019). Experimental manipulation of *N. attenuata* flowers from an upright (i.e. 45^{o} angle) to that of a downward facing orientation (i.e. -45^{o} angle), resulted in the complete negation of seed production when visited by naïve *M. sexta* hawkmoths (Yon et al., 2017). Similarly, manipulation of *N. attenuata* flowers into a downward orientation (-45^{o}) resulted in a significant reduction in pollen delivery, virtually negated pollen removal, and resulted in a significantly lower foraging success for the moths (Haverkamp et al., 2019).

Scent supplementation of model flowers did appear to slightly increase visitation to targeted model flowers (Figure 8.). In other studies, colour has been shown to take priority over scent as a signalling role in butterfly advertisement, whereby the experimental addition of scent to less appealing targets (i.e. not innately preferred colours), increased attraction in *Vanessa indica* (Ômura and Honda, 2005). *Vanessa indica* visitation to yellow targets (an innate preference), did not differ between those that are scented or scentless, indicating that olfactory information is redundant in the presence of innate visual preference cues (Ômura and Honda, 2005). *Papilio nireus lyaeus* brushed scented models significantly more than scented models in this study, whereas no clear preference was observed with regards to other butterfly species. Scent-supplementation to real inflorescences greatly increased the number of *P. dardanus cena* approach and brush visits compared to unscented controls (Figure 11). Scent is an important signalling cue and is able to attract pollinators at large distances (Metcalf and Metcalf, 1992), enhancing pollinator specificity (Groth, Bergstrom, and Pellmyr, 1987; Whitten and Williams, 1992; Mant, Peakall, and Schiestl, 2005) as well as pollinator effectiveness and reliability (Raguso et al., 2003).

Papilio dardanus cena of both sexes and *P. demodocus* males showed clear EAD responses to benzaldehyde (Figure 12A). More than half of the plant families studied so far contain benzaldehyde (Knudsen and Gershenzon, 2006), suggesting that this may be a signal used by numerous species for attraction, however no patterns have yet emerged linking floral scents with butterfly pollination (Andersson et al., 2002). It has however been speculated that the extensive frequency of the monoterpenes such as the linalool group and the benzenoids 2-phenylethanol and phenylacetaldehyde may represent traits characteristic of a butterfly pollination syndrome (Andersson et al., 2002). Both female *P. dardanus cena* and male *P. demodocus* showed antennal responses to benzyl alcohol and benzyl benzoate, whilst females showed clear responses to benzaldehyde, benzyl alcohol and benzyl benzoate (Figure 12B & C). In the butterfly *Heliconius melpomene*, females have been show to produce stronger antennal responses than males (Andersson and Dobson, 2003a). Examination of the olfactory cues deployed by *Brassica rapa* L. in the attraction of *P. rapae*, has demonstrated strong responses in PER to phenylacetaldehyde and moderate responses to benzaldehyde, benzyl alcohol, 2-phenylethanol and phenylacetonitrile, with similar EAG responses at higher doses (Ômura, Honda, and Hayashi, 1999). Results from this study suggest that these floral volatiles could facilitate location and floral recognition at close- rather than long-ranges (Ômura, Honda, and Hayashi, 1999). This may explain increased brush visits by *P. nireus lyaeus* to both scented models and live flowers. *Vanessa indica* has also shown strong PER in response to benzaldehyde, but overall the butterflies relied primarily on colour and used scent as a secondary cue for floral visitation (Ômura and Honda, 2005). *Heliconius melpomene* similarly, has been shown to produce strong EAD responses to benzaldehyde and uses visual cues when choosing flowers, whilst scent initiates and maintains floral foraging (Andersson and Dobson, 2003b). Biotic pollination has been closely linked to scented flowers (Farré -Armengol et al., 2015), but much work is required to understand the role of scent in butterfly pollination, particularly in south African systems. Scent appears to elicit illicit attraction in some species and indifference in others. Benzaldehyde appears to present a commonality in attraction, but more work is required to fully understand the role of scent in *C. miniata*.

CONCLUSIONS AND PERSPECTIVES

Our initial hypothesis that butterflies favour orange flowers with a central yellow target pattern over other colours and patterns was generally supported (Figure 4, 5). In paired tests (Figure 5), orange flowers with a central yellow target were overwhelmingly favoured. Our second hypothesis that butterflies will be more attracted to, and more likely to settle on, flowers which face upwards was partially supported due to the fact that no attraction bias in approach and brush visits to pendulously manipulated flowers was found. *Papilio dardanus cena* nevertheless strongly preferred to alight on upright models. Our third hypothesis, that butterflies will show electrophysiological responses to scent compounds emitted by *C. miniata* and prefer scented over unscented flowers, was supported. Although attraction bias to scent-supplemented model arrays was not strong (Figure 8), approach and brush visits to real flowers were significantly increased by the addition of scent (Figure 11), indicating that scent in combination with other visual or tactile traits of real flowers increased attraction. EAD responses to odour compounds identified benzaldehyde as a common stimulus in at least two swallowtail species, highlighting its role as a signalling cue.

Floral traits of *Clivia miniata* appeal to a wide variety of butterflies, belonging to three families. Orange colouration is likely a reflection of a degree of innate pollinator preference for this colour, but butterfly conditioning to prefer orange cannot be ruled out. As in many other butterfly-pollinated species, colour plays a primary role as an advertising cue in *C. miniata*. Butterflies favoured plain orange over yellow colours, but orange models with yellow targets were significantly more attractive than plain orange models. Importantly, the orange colouration of *C. miniata* var. *miniata* was favoured over the yellow of *C. miniata* var. *citrina.* Upright floral orientation plays a pivotal role in facilitating butterfly visitation. The evolution of upright from pendulous orientation, and a widening of the corolla in the common ancestor of *C. miniata* was likely the first and most vital floral trait modifications taking place during the putative shift from bird- to butterfly-pollination in *Clivia* (Kiepiel and Johnson, 2014). Upright orientation likely provided an approach vector and platform for landing, whilst the development of trumpet-shaped flowers would have facilitated wing pollination. The combination of colour and scent create a stronger advertising signal, making model and real flowers alike more attractive. Scent elicited the most variable response, although responses to benzaldehyde were detectable in at least two swallowtail species. The advent of scent was likely one of the latter floral trait modifications, and one which would entice butterflies at a longer range distance and possibly also attract a wider array of butterflies. This research underpins the scarcity of work conducted on signal perception in southern African butterflies, highlighting the work required to illuminate innate colour preferences, conditioning and learning, in order to decipher the role of pollinator-mediated selection in the evolution of butterfly pollination.

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REFERENCES

ANDERSSON, S., AND H. E. M. DOBSON. 2003a. Antennal responses to floral scents in the butterfly *Heliconius melpomene*. *Journal of Chemical Ecology* 29: 2319-2330.

------. 2003b. Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. *Journal of Chemical Ecology* 29: 2303-2318.

ANDERSSON, S., L. A. NILSSON, I. GROTH, AND G. BERGSTROM. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Botanical Journal of the Linnean Society* 140: 129-153.

ARIKAWA, K., AND M. KINOSHITA. 2000. Colour constancy of the swallowtail butterfly *Papilio xuthus*. *Journal of Experimental Biology* 203: 3521-3530.

ARMBRUSTER, W. S. 1996. Evolution of floral morphology and function: an integrative approach to adaptation, constraint, and compromise in *Dalechampia* (Euphorbiaceae). *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal-pollinated plants, 241-272. Chapman and Hall, New York, USA.

ARROYO, M. K., I. TILL-BOTTRAUD, C. TORRES, C. A. HENRIQUEZ, AND J. MARTINEZ. 2007. Display size preferences and foraging habits of high Andean butterflies pollinating *Chaetanthera lycopodioides* (Asteraceae) in the subival of the central Chilean Andes. *Arctic, Antarctic and Alpine Research* 39: 347-352.

BALKENIUS, A., AND M. DACKE. 2010. Flight behaviour of the hawkmoth *Manduca sexta* towards unimodal and multimodal targets. *Journal of Experimental Biology* 213: 3741–3747.

BELL, G. 1985. On the function of flowers. *Proceedings of the Royal Society of London, Series B, Biological Science* 224: 223-265.

BERGSTRÖM, G., H. E. M. DOBSON, AND I. GROTH. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). *Plant Systematics and Evolution* 195: 221-242.

BLACKISTON, D. J., A. D. BRISCOE, AND M. R. WEISS. 2011. Colour vision and learning in the monarch butterfly, *Danaus plexippus* (Nymphalidae). *Journal of Experimental Biology* 214: 509-520.

BRADSHAW, H. D., AND D. W. SCHEMSKE. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426: 176-178.

BRISCOE, A. D., AND L. CHITTKA. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46: 471-510.

CAMPBELL, D. R. 1989. Measurements of selection in a hermaphroditic plant: variation in male and female pollination success. *Evolution* 43: 318-335.

------. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103: 1557-1566.

CAMPBELL, D. R., N. M. WASER, AND E. J. MELÉNDEZ-ACKERMAN. 1997. Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *American Naturalist* 149: 295-315.

CAMPBELL, D. R., A. JÜRGENS, AND S. D. JOHNSON. 2016. Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices. *New Phytologist* 210: 333-342.

CAMPOS, E. O., H. D. BRADSHAW, AND T. L. DANIEL. 2015. Shape matters: corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. *Functional Ecology* 29: 462-468.

CARUSO, C. M., K. E. EISEN, R. A. MARTIN, AND N. SLETVOLD. 2019. A meta-analysis of the agents of selection on floral traits. *Evolution* 73: 4–14.

CASPER, B. B., AND T. R. LA PINE. 1984. Changes in corolla color and other floral characteristics in *Cryptantha humilis* (Boraginaceae): cues to discourage pollinators? *Evolution* 38: 128-141.

CEPERO, L. C., L. C. ROSENWALD, AND M. R. WEISS. 2015. The relative importance of flower colour and shape for the foraging monarch butterfly (Lepidoptera: Nymphalidae) *Journal of Insect Behavior* 28: 499-511.

CHITTKA, L., AND N. E. RAINE. 2006. Recognition of flowers by pollinators. *Current Opinion in Plant Biology* 9: 428-435.

CHITTKA, L., J. D. THOMSON, AND N. M. WASER. 1999. Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86: 361-377.

CHITTKA, L., A. SHMIDA, N. TROJE, AND R. MENZEL. 1994. Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Research* 34: 1489-1508.

CLEMENTS, F. E., AND F. L. LONG. 1923. Experimental pollination - an outline of the ecology of flowers and insects. Carnegie Institution of Washington, Washington, DC, USA.

CONRAD, F., G. REEVES, D. A. SNIJMAN, AND T. A. J. HEDDERSON. 2006. Genetic relationships within the tribe Haemantheae (Amaryllidaceae) based on plastid DNA sequence data. *In* S. A. Ghazanfar AND H. J. Beentje [eds.], Taxonomy and ecology of African plants, their conservation and sustainable use, 73-83. Royal Botanic Gardens Kew, UK.

CRANE, J. 1955. Imaginal behavior of a Trinidad butterfly, *Heliconius erato hydara* Hewitson, with special reference to the social use of color. *Zoologica* 40: 167-196.

DAFNI, A., AND P. G. KEVAN. 1997. Flower size and shape: implications in pollination. *Israel Journal of Plant Sciences* 45: 201-212.

DAFNI, A., AND M. GIURFA. 1999. The functional ecology of floral guides in relation to insects behaviour and vision. *In* S. P. Wasser [ed.], Evolutionary theory and processes: Modern perspectives, 363-383. Springer, Dordrecht, Netherlands.

DARWIN, C. 1877. The Different Forms of Flowers on Plants of the Same Species. John Murray, London, UK.

DARWIN, C. R. 1862. On the various contrivances by which British and foreign orchids are fertilized by insects. John Murray, London, UK.

DAUMER, K. 1958. Blumenfarben, wie sie die bienen sehen. *Zeitschrift fuer Vergleichende Physiologie* 41: 49-110.

DE LOS MOZOS PASCUAL, M., AND L. M. DOMINGO. 1991. Flower constancy in *Heliotaurus ruficollis* Fabricius, 1781 (Coleoptera: Allestancy). *Elytron* 5: 9-11.

DINKEL, T., AND K. LUNAU. 2001. How drone flies (*Eristalis tenax* L., Syrphidae, Diptera) use floral guides to locate food sources. *Journal of Insect Physiology* 47: 1111-1118.

DOBSON, H. E. M. 2006. Relationship between floral fragrance composition and type of pollinator. *In* N. Dudareva AND E. Pichersky [eds.], Biology of floral scents, 147-198. Taylor & Francis, Boca Raton, USA.

DORNHAUS, A., AND L. CHITTKA. 1999. Insect behaviour: evolutionary origins of bee dances. *Nature* 401: 38-38.

ELLIS, A. G., AND S. D. JOHNSON. 2012. Lack of floral constancy by bee fly pollinators: implications for ethological isolation in an African daisy. *Behavioral Ecology* 23: 729-734.

ENDLER, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. *Biological Journal of the Linnean Society* 41: 315-352.

FAEGRI, K., AND L. VAN DER PIJL. 1979. The principles of pollination ecology. Pergamon, Oxford, UK.

FARRÉ -ARMENGOL, G. F., I. FILELLA, J. LLUSIÀ, AND J. PENUELAS. 2015. Pollination mode determines floral scent. *Biochemical Systematics and Ecology* 61: 44-53.

FENSTER, C. B., W. S. ARMBRUSTER, AND M. R. DUDASH. 2009. Specialization of flowers: is floral orientation an overlooked first step? *New Phytologist* 183: 502-506.

FOX, R. M. 1966. Forelegs of butterflies. *Journal of Research on the Lepidoptera* 5: 1-12.

FULTON, M., AND S. A. HODGES. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society of London, Series B, Biological Science* 266: 2247-2252.

GERVASI, D. D. L., AND F. P. SCHIESTL. 2017. Real-time divergent evolution in plants driven by pollinators. *Nature Communications* 8: 14691.

GIURFA, M., A. DAFNI, AND P. R. NEAL. 1999. Floral symmetry and its role in plant-pollinator systems. *International Journal of Plant Sciences* 160: S41-S50.

GOLDBLATT, P., AND J. C. MANNING. 2002. Evidence for moth and butterfly pollination in *Gladiolus* (Iridaceae-Crocoideae). *Annals of the Missouri Botanical Garden* 89: 110-124.

GOODALE, E., E. KIM, A. NABORS, S. HENRICHON, AND J. C. NIEH. 2014. The innate responses of bumble bees to flower patterns: separating the nectar guide from the nectary changes bee movements and search time. *Naturwissenschaften* 101: 523-526.

GOULD, J. L., AND P. MARLER. 1984. Ethology and the natural history of learning. *In* P. Marler AND H. S. Terrace [eds.], The biology of learning. Dahlem Workshop Reports (Life Sciences Research Reports), vol. 209. Springer, Berlin, Germany.

GOULSON, D., AND J. S. CORY. 1993. Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecological Entomology* 18: 315-320.

GOULSON, D., AND N. P. WRIGHT. 1998. Flower constancy in the hoverflies *Episyrphus balteatus* (Degeer) and *Syrphus ribesii* (L.) (Syrphidae). *Behavioral Ecology* 9: 213-219.

GOULSON, D., J. OLLERTON, AND C. SLUMAN. 1997. Foraging strategies in the small skipper buttefly, *Thymelicus flavus*: When to switch? *Animal Behaviour* 53: 1009-1016.

GOULSON, D., J. C. STOUT, AND S. A. HAWSON. 1997. Can flower constancy in nectaring butterflies be explained by Darwin's interference hypothesis? *Oecologia* 112: 225-231.

GOULSON, D., J. C. STOUT, S. A. HAWSON, AND J. A. ALLEN. 1998. Floral display size in comfrey, *Symphytum officinale* L. (Boraginaceae): relationships with visitation by three bumblebee species and subsequent seed set. *Oecologia* 113: 502-508.

GOYRET, J., AND R. A. RAGUSO. 2006. The role of mechanosensory input in flower handling efficiency and learning by *Manduca sexta*. *Journal of Experimental Biology* 209: 1585-1593.

GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82-97.

------. 1950. The flower constancy of bees. *Botanical Review* 16: 379-398.

GRANT, V., AND K. A. GRANT. 1965. Flower pollination in the Phlox family. Columbia University Press, New York, USA.

GRINDELAND, J. M., N. SLETVOLD, AND R. A. IMS. 2005. Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. *Functional Ecology* 19: 383-390.

GROSS, C. L. 1992. Floral traits and pollinator constancy: foraging by native bees among three sympatric legumes. *Journal of Ecology* 17: 67-74.

GROTH, I., G. BERGSTROM, AND O. PELLMYR. 1987. Floral fragrances in *Cimicifuga*: chemical polymorphism and incipient speciation in *Cimicifuga simplex*. *Biochemical Systematics and Ecology* 15: 441-444.

GUO, X., W. PAN, J. E. CONNETT, P. J. HANNAN, AND S. A. FRENCH. 2005. Small-sample performance of the robust score test and its modifications in generalized estimating equations. *Statistics in Medicine* 24: 3479-3495.

HANSEN, D. M., T. VAN DER NIET, AND S. D. JOHNSON. 2012. Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proceedings of the Royal Society B* 279: 634-639.

HARDER, L. D., AND S. D. JOHNSON. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist* 183: 530-545.

HAVERKAMP, A., X. LI, B. S. HANSSON, I. T. BALDWIN, M. KNADEN, AND F. YON. 2019. Flower movement balances pollinator needs and pollen protection. *Ecology* 100: e02553.

HILL, P. S. M., P. H. WELLS, AND H. WELLS. 1997. Spontaneous flower constancy and learning in honey bees as a function of colour. *Animal Behaviour* 54: 615-627.

HIROTA, S. K., N. MIKI, A. A. YASUMOTO, AND T. YAHARA. 2019. UV bullseye contrast of *Hemerocallis* flowers attracts hawkmoths but not swallowtail butterflies *Ecology and Evolution* 9: 52-64.

HIROTA, S. K., K. NITTA, Y. KIM, N. KAWAKUBO, A. A. YASUMOTO, AND T. YAHARA. 2012. Relative role of flower colour and scent on pollinator attraction: Experimental tests using F1 and F2 hybrids of daylily and nightlily. *PLOS ONE* 7: e39010.

HODGES, S. A. 2002. Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *American Naturalist* 159: s51-s60.

HODGES, S. A., AND M. L. ARNOLD. 1994a. Columbines: A geographically widespread species flock. *Proceedings of the National Academy of Science, USA* 91: 5219-5132.

HODGES, S. A., AND M. L. ARNOLD. 1994b. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences, USA* 91: 2493-2496.

HUBER, F. K., R. KAISER, W. SAUTER, AND F. P. SCHIESTL. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* 142: 564-575.

ILSE, D. 1928. Uber den Farbensinn der Tagfalter. *Z. f. vergl. Physiologie Bd.* 8: 658-692.

ILSE, D., AND V. G. VAIDYA. 1956. Spontaneous feeding response to colours in *Papilio demoleus* L. *Proceedings of the Indian Academy of Science, Section B, Plant Science* 43: 23-31.

JERSÁKOVÁ, J., A. JÜRGENS, P. ŠMILAUER, AND S. D. JOHNSON. 2012. The evolution of floral mimicry: identifying traits that visually attract pollinatiors. *Functional Ecology* 26: 1381-1389.

JOHNSON, S. D. 1994. Evidence for Batesian mimicry in a butterfly-pollinated orchid. *Biological Journal of the Linnean Society* 53: 91-104.

JOHNSON, S. D., AND W. J. BOND. 1994. Red flowers and butterfly pollination in the fynbos of South Africa. *In* M. Arianoutsou AND R. H. Groves [eds.], Plant-animal interactions in Mediterranean-type ecosystems, 137-148. Kluwer Academic Publishers, Dordrecht, Netherlands.

JOHNSON, S. D., AND A. DAFNI. 1998. Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Functional Ecology* 12: 289-297.

JOHNSON, S. D., AND S. ANDERSSON. 2002. A simple field method for manipulating ultraviolet reflectance of flowers. *Canadian Journal of Botany* 80: 1325-1328.

JOHNSON, S. D., AND P. WESTER. 2017. Stefan Vogel's analysis of floral syndromes in the South African flora: An appraisal based on 60 years of pollination studies. *Flora* 232: 200-206.

JURGENS, A., T. WITT, AND G. GOTTSBERGER. 2003. Flower scent composition in *Dianthus* and *Saponaria* species (Caryophyllaceae) and its relevance for pollination biology and taxonomy. *Biochemical Systematics and Ecology* 31: 345-357.

KACZOROWSKI, R. L., A. R. SELIGER, A. C. GASKETT, S. K. WIGSTEN, AND R. A. RAGUSO. 2012. Corolla shape vs. size in flower choice by a nocturnal hawkmoth pollinator. *Functional Ecology* 26: 577-587.

KANDORI, I., AND N. OHSAKI. 1996. The learning abilities of the white cabbage butterfly, *Pieris rapae*, foraging for flowers. *Researches on Population Ecology* 38: 111-117.

------. 1998. Effect of experience on foraging behavior towards artificial nectar guide in the cabbage butterfly, *Pieris rapae crucivora* (Lepidoptera: Pieridae). *Applied Entomology and Zoology* 33: 35-42.

KANDORI, I., AND T. YAMAKI. 2012. Reward and non-reward learning of flower colours in the butterfly *Byasa alcinous* (Lepidoptera: Papilionidae). *Naturwissenschaften* 99: 705–713.

KANDORI, I., T. YAMAKI, S.-I. OKUYAMA, N. SAKAMOTO, AND T. YOKOI. 2009. Interspecific and intersexual learning rate differences in four butterfly species. *Journal of Experimental Biology* 212: 3810-3816.

KEASAR, T., Y. BILU, U. MOTRO, AND A. SHMIDA. 1997. Foraging choices of bumblebees on equally rewarding artificial flowers of different colors. *Israel Journal of Plant Sciences* 45: 223-233.

KEVAN, P. G. 1983. Floral colors through the insect eye: what are they and what do they mean? *In* C. E. Jones AND R. J. Little [eds.], Handbook of experimental pollination biology, 3-30. Van Nostrand Reinhold, New York, USA.

KEVAN, P. G., AND H. G. BAKER. 1983. Insects as flower visitors and pollinators. *Annual Review of Entomology* 28: 407-453.

KIEPIEL, I., AND S. D. JOHNSON. 2014. Shift from bird to butterfly pollination in *Clivia* (Amaryllidaceae). *American Journal of Botany* 101: 190-200.

KINOSHITA, M., AND K. ARIKAWA. 2014. Colour and polarization vision in foraging *Papilio*. *Journal of Comparative Physiology A* 200: 513-526.

KINOSHITA, M., N. SHIMADA, AND K. ARIKAWA. 1999. Colour vision of the foraging swallotail butterfly *Papilio xunthus*. *The Journal of Experimental Biology* 202: 95-102.

KINOSHITA, M., F. J. STEWART, AND H. ÔMURA. 2017. Multisensory integration in Lepidoptera: Insights into flower-visitor interactions. *BioEssays* 39: 1600086.

KLINKHAMER, P. G. L., AND T. J. DE JONG. 1990. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). *Oikos* 57: 399-405.

KNUDSEN, J. T., AND J. GERSHENZON. 2006. The chemical diversity of floral scent. *In* N. Dudareva AND E. Pichersky [eds.], Biology of floral scent, 27-52. Taylor & Francis, Boca Raton, USA.

KOSHITAKA, H., K. ARIKAWA, AND M. KINOSHITA. 2011. Intensity contrast as a crucial cue for butterfly landing. *Journal of Comparative Physiology A* 197: 1105-1112.

KOSHITAKA, H., M. KINOSHITA, M. VOROBYEV, AND K. ARIKAWA. 2008. Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proceedings of the Royal Society B* 275: 947-954.

KUGLER, H. 1943. Hummeln als blütenbesucher. *Ergebenisse der Biologie* 19: 143-323.

KUGLER, H. 1963. UV-musterungen auf blüten und ihr zustandekommen. *Planta* 59: 296-329.

LAWSON, D. A., H. M. WHITNEY, AND S. A. RANDS. 2017. Nectar discovery speeds and multimodal displays: assessing nectar search times in bees with radiating and non-radiating guides. *Evolutionary Ecology* 31: 899-912.

LEONARD, A. S., AND D. R. PAPAJ. 2011. 'X' marks the spot: The possible benefits of nectar guides to bees and plants. *Functional Ecology* 25: 1293-1301.

LEONARD, A. S., AND P. MASEK. 2014. Multisensory integration of colors and scents: insights from bees and flowers. *Journal of Comparative Physiology A* 200: 463-474.

LEWIS, A. C. 1986. Memory constraints and flower choice in *Pieris rapae*. *Science* 232: 863-865.

LEWIS, A. C. 1989. Flower visit consistency in *Pieris rapae*, the cabbage butterfly. *Journal of Animal Ecology* 58: 1-13.

LEWIS, A. C., AND G. A. LIPANI. 1990. Learning and flower use in butterflies: Hypotheses from honey bees. *In* E. A. Bernays [ed.], Insect-plant interactions, 95-110. CRC Press, Boca Raton, USA.

LUNAU, K. 1990. Colour saturation triggers innate reactions to flower signals: flower dummy experiments with bumblebees. *Journal of Comparative Physiology A* 166: 827-834.

LUNAU, K., AND E. J. MAIER. 1995. Innate colour preferences of flower visitors. *Journal of Comparative Physiology A* 177: 1-19.

LUNAU, K., G. FIESELMANN, B. HEUSCHEN, AND A. VAN DE LOO. 2006. Visual targeting of components of floral colour patterns in flower-naive bumblebees (*Bombus terrestris*; Apidae) *Naturewissenschaften* 93: 325-328.

MAKINO, T. T., K. OHASHI, AND S. SAKAI. 2007. How do floral display size and the density of surrounding flowers influence the likelihood of bumble bee revisitation to a plant. *Functional Ecology* 21: 87-95.

MANT, J., R. PEAKALL, AND F. P. SCHIESTL. 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? *Evolution* 59: 1449-1463.

MELÉNDEZ-ACKERMAN, E. J., D. R. CAMPBELL, AND N. M. WASER. 1997. Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* 78: 2532-2541.

MENZEL, R. 1979. Behavioral access to short-term memory in bees. *Nature* 281: 368-369.

MENZEL, R., AND A. SHMIDA. 1993. The ecology of flower colors and the natural colour vision of insect pollinators: The Israeli flora as a study case. *Biological Reviews* 68: 81-120.

METCALF, R. L., AND E. R. METCALF. 1992. Plant kairomones in insect ecology and control. Chapman & Hall, New York, USA.

MICO, E., AND E. GALANTE. 2005. Behavior of Cetonid beetles (Coleoptera: Scarabaeoidea: Cetoniidae) on the Mediterranean shrub *Cistus albidus Elytron* 19: 89–98.

MITCHELL-OLDS, T., AND R. G. SHAW. 1987. Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution* 41: 1149-1161.

MITCHELL, R. J., J. D. KARRON, K. G. HOLMQUIST, AND J. M. BELL. 2004. The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology* 18: 116-124.

MULLIGAN, G. A., AND P. G. KEVAN. 1973. Color, brightness, and other floral characteristics attracting insects to the blossoms of some Canadian weeds. *Canadian Journal of Botany* 51: 1939-1952.

OHARA, M., AND S. HIGASHI. 1994. Effects of inflorescence size on visits from pollinators and seed set of *Corydalis ambigua* (Papaveraceae). *Oecologia* 98: 25-30.

ÔMURA, H., AND K. HONDA. 2005. Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* 142: 588-596.

ÔMURA, H., K. HONDA, AND N. HAYASHI. 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. *Journal of Chemical Ecology* 25: 1895-1906.

PENNY, J. H. J. 1983. Nectar guide color contrast: a possible relationship with pollination strategy. *New Phytologist* 95: 707-721.

POHL, N. B., J. VAN WYK, AND D. R. CAMPBELL. 2011. Butterflies show flower colour preferences but not constancy in foraging at four plant species. *Ecological Entomology* 36: 290-300.

POLICHA, T., A. DAVIS, M. BARNADAS, B. T. DENTINGER, R. A. RAGUSO, AND B. A. ROY. 2016. Disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic three-dimensional printed flowers. *New Phytologist* 2100: 1058-1071.

RAGUSO, R. 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology and Systematics* 39: 549-569.

RAGUSO, R. A. 2004. Why are some floral nectars scented? *Ecology* 85: 1486-1494.

RAGUSO, R. A., AND M. A. WILLIS. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. *Animal Behaviour* 64: 685-695.

------. 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Animal Behaviour* 69: 407-418.

RAGUSO, R. A., R. A. LEVIN, S. E. FOOSE, M. W. HOLMBERG, AND L. A. MCDADE. 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry* 63: 265-284.

RAIMONDO, D., L. VON STADEN, W. FODEN, J. E. VICTOR, N. A. HELME, R. C. TURNER, D. A. KAMUNDI, AND P. A. MANYAMA. 2009. Red List of South African plants. South African National Biodiversity Institute, Pretoria, South Africa.

RAINE, N. E., AND L. CHITTKA. 2005. Comparison of flower constancy and foraging performance in three bumblebee species (Hymenoptera: Apidae: *Bombus*). *Entomologia Generalis* 28: 81-89.

ROBERTSON, A. W., AND M. R. MACNAIR. 1995. The effects of floral display size on pollinator service to individual flowers of *Myosotis* and *Mimulus*. *Oikos* 72: 106-114.

RODRIGUEZ-GIRONES, M. A., AND L. SANTAMARIA. 2004. Why are so many bird flowers red? *Plos Biology* 2: 1515-1519.

SCHEMSKE, D. W., AND J. ÅGREN. 1995. Deceit pollination and selection on female flower size in *Begonia involucrata*: an experimental approach. *Evolution* 49: 207-214.

SCHEMSKE, D. W., AND H. D. BRADSHAW, JR. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus). Proceedings of the National Academy of Science, USA* 96: 11910-11915.

SCHERER, C., AND G. KOLB. 1987. Behavioral experiments on the visual processing of color stimuli in *Pieris brassicae* L. (Lepidoptera). *Journal of Comparative Physiology A* 160: 645-656.

SCHIESTL, F. P., AND S. DÖTTERL. 2012. The evolution of floral scent and olfactory preferences in pollinators: coevolution or pre-existing bias? *Evolution* 66: 2042-2055.

SCHIESTL, F. P., AND S. D. JOHNSON. 2013. Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution* 28: 307-315.

SHUTTLEWORTH, A., S. D. JOHNSON, AND A. JURGENS. 2017. Entering through the narrow gate: A morphological filter explains specialized pollination of a carrion-scented stapeliad. *Flora* 232: 92-103.

SILBERGLIED, R. E. 1984. Visual communication and sexual selection among butterflies. *In* R. I. Vane-Wright AND P. R. Ackery [eds.], The biology of butterflies, 207-233. Academic Press, London, UK.

SLETVOLD, N., AND J. ÅGREN. 2010. Pollinator-mediated selection on floral display and spur length in the orchid *Gymnadenia conopsea*. *International Journal of Plant Sciences* 171: 999-1009.

SLETVOLD, N., J. TRUNSCHKE, M. SMIT, J. VERBEEK, AND J. ÅGREN. 2016. Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70: 716-724.

SPRENGEL, C. K. 1793. Das entdeckte geheimnifs der natur in bau un in der befruchtung der blumen. Friedrich Vieweg dem aeltern, Berlin, Germany.

STEBBINS, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307-326.

SWIHART, C. A. 1971. Colour discrimination by the butterfly *Heliconius charitonius* Linn. *Animal Behaviour* 19: 156-164.

SWIHART, C. A., AND S. L. SWIHART. 1970. Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius* Linn. *Animal Behaviour* 18: 60-64.

SWIHART, S. L. 1970. The neural basis of colour vision in the butterfly, *Papilio troilus*. *Journal of Insect Physiology* 16: 1623-1636.

TADEY, M., AND M. A. AIZEN. 2001. Why do flowers of a hummingbird-pollinated mistletoe face down? *Functional Ecology* 15: 782-790.

TAKEDA, K. 1961. The nature of impulses of single tarsal chemoreceptors in the butterfly *Vanessa indica*. *Journal of Cellular and Comparative Physiology* 58: 233-245.

USHIMARU, A., AND F. HYODO. 2005. Why do bilaterally symmetrical flowers orient vertically? Flower orientation influences pollinator landing behaviour. *Evolutionary Ecology Research* 7: 252-260.

USHIMARU, A., D. KAWASE, AND A. IMAMURA. 2006. Flowers adaptively face down-slope in 10 forest-floor herbs. *Functional Ecology* 20: 585-591.

VAN DER NIET, T., AND S. D. JOHNSON. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* 27: 353-361.

VAUGHTON, G., AND M. RAMSEY. 1998. Floral display, pollinator visitation and reproductive success in the dioecious perennial herb *Wurmbea dioica* (Liliaceae). *Oecologia* 115: 93-101.

VRIELING, K., P. SAUMITOU-LAPRADE, J. CUGUEN, H. VAN DIJK, T. J. DE JONG, AND P. G. L. KLINKHAMER. 1999. Direct and indirect estimates of the selfing rate in small and large individuals of the bumblebee pollinated *Cynoglossum officinale* L. (Boraginaceae). *Ecology Letters* 2: 331-337.

WANG, H., C.-L. XIAO, R. W. GITURU, Z. XIONG, D. YU, Y.-H. GUO, AND C.-H. YANG. 2014. Change of floral orientation affects pollinator diversity and their relative importance in an alpine plant with generalized pollination system, *Geranium refractim* (Geraniaceae). *Plant Ecology* 215: 1211-1219.

WASER, N. M. 1983. The adaptive nature of floral traits: ideas and evidence. *In* L. Real [ed.], Pollination biology, 241-285. Academic Press, Orlando, USA.

------. 1986. Flower constancy: definition, cause, and measurement. *American Naturalist* 127: 593-603.

------. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. *Oikos* 82: 198-201.

WASER, N. M., AND M. V. PRICE. 1983. Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302: 422-424.

WASER, N. M., AND M. V. PRICE. 1985. The effect of nectar guides on pollinator preference: experimental studies with a montane herb. *Oecologia* 67: 121-126.

WEISS, M. R. 1995a. Floral color change: a widespread functional convergence. *American Journal of Botany* 82: 167-185.

------. 1995b. Associative colour learning in a nymphalid butterfly. *Ecological Entomology* 20: 298-301.

------. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Animal Behaviour* 53: 1043-1052.

WEISS, M. R. 2001. Vison and learning in some neglected pollinators: beetles, flies moths and butterflies. *In* L. Chittka AND J. D. Thomson [eds.], Cognitive ecology of pollination: animal behaviour and floral evolution, 171-190. Cambridge University Press, Cambridge, UK.

WEISS, M. R., AND D. R. PAPAJ. 2003. Colour learning in two behavioural contexts: how much can a butterfly keep in mind? *Animal Behaviour* 65: 425-434.

WHITTEN, W. M., AND N. H. WILLIAMS. 1992. Floral fragrances of *Stanhopea* (Orchidaceae). *Lindleyana* 7: 130-153.

WILSON, M. F., AND P. W. PRICE. 1977. The evolution of inflorecence size in *Asclepias* (Asclepiadaceae). *Evolution* 31: 495-511.

WILSON, P., AND M. STINE. 1996. Floral constancy in bumble bees: handling efficiency or perceptual conditioning? *Oecologia* 106: 493-499.

YON, F., D. KESSLER, Y. JOO, L. CORTÉS LLORCA, S.-G. KIM, AND I. T. BALDWIN. 2017. Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*. *Journal of Integrative Plant Biology* 59: 180-189.

YOSHIDA, M., Y. ITOH, H. ÔMURA, K. ARIKAWA, AND M. KINOSHITA. 2015. Plant scents modify innate colour preference in foraging swllowtail butterflies. *Biology Letters* 11: 20150390.

ZHANG, C., N. J. VEREECKEN, L. WANG, B. TIAN, A. DAFNI, Y. YANG, AND Y. DUAN. 2017. Are nectar guide colour changes a reliable signal to pollinators that enhance reproductive success? *Plant Ecology & Diversity* 10: 89-96.

ZUUR, A. F., E. N. IENO, N. J. WALKER, A. A. SAVELIEV, AND G. M. SMITH. 2009. Mixed model effects and extensions in ecoloy with R. 1st Edition. Springer, New York, USA.

Figure legends

Figure 1. (A) *Papilio dardanus cena* (male) exhibiting typical approach behaviour prior to alighting on a *C. miniata* inflorescence at the UNR site. (B) *Papilio dardanus cena* (male) carrying pollen on wings, probing a *C. miniata* flower at the MPNR site. (C) '*Clivia miniata*' paper model used in choice tests (here orange with yellow target), with vial containing artificial floral scent. (D) *Papilio dardanus cena* (male) attempting unsuccessfully to alight on pendulous orientated *C. miniata* flower during choice tests at the UNR site. (E) *Papilio dardanus cena* males feeding on emasculated *C. miniata* var. *citrina* inflorescence during paired choice tests at the UNR site.

Figure 2. (A) Spectral reflectance of *C. miniata* and *C. miniata* var. *citrina* flowers as well as model paper used in artificial flower arrays (sample size as below, sans *C. gardenii*). (B) Segment classification according to Endler (1990) of *C. miniata, C. miniata* var. *citrina* and *C. gardenii* floral spectra, as well as model paper spectra. Spectra illustrate mean reflectance for real and model flowers, where *n* represents the number of samples, each taken from a separate plant or sheet of paper respectively. *Clivia miniata* (MPNR; *n* = 12, UNR; *n* = 10), *C. miniata* var. *citrina* (*n* = 10), *C. gardenii* (Pondoland; *n* = 33, UNR; *n* = 13, GG; *n* = 8), model paper (*n* = 6, for all 6 colours).

Figure 3. Behavioural responses of *P. dardanus cena* to model arrays consisting of five plain (unpatterned) colour choices. Experimental arrays conducted at the UNR site in the flowering season of 2013. Observations (*n* = 121) recorded over 5 full days, and over 26 unique time blocks. Approaches; *n* = 99, brushes; *n* = 16; alights; *n* = 6.

Figure 4. Behavioural responses of five butterfly species (*N. argia, P. euphranor, P. dardanus cena, P. ophidicephalus, P. echerioides echerioides*) to model flower arrays consisting of six *C. miniata*-shaped choices (three plain colours and three with targets). Colours from left to right are: yellow; orange; red; yellow with red target; orange with yellow target; red with yellow target. Experimental arrays conducted at the MPNR site in October 2010. Observations (*n* = 121) recorded over 2 full days, and over 8 unique time blocks. Approaches; *n* = 70, brushes; *n* = 26, alights; *n* = 25.

Figure 5. Behavioural responses of *P. dardanus cena* to two *C. miniata*-shaped model flower arrays; plain orange versus orange with a yellow target. Experimental arrays conducted at the UNR site in the flowering season of 2013. Observations (*n* = 125) recorded over 5 full days, and over 45 unique time blocks. Approaches; *n* = 113; brushes *n* = 12; alights *n* = 0.

Figure 6. Behavioural responses of *P. ophidicephalus* to two *C. miniata*-shaped model choices, differing only in size; plain orange versus plain orange reduced-size (one-third scale) models. Experimental

arrays conducted at the MPNR site in October 2010. Observations (*n* = 32) recorded over 4 full days, and over 10 unique time blocks. Approaches; *n* = 28; brushes; *n* = 2, alights; *n* =2.

Figure 7. Behavioural responses of five butterfly species (*N. argia*, *P. echerioides echerioides*, *P. euphranor*, *P. nireus lyaeus* and *P. ophidicephalus*) to two model flower arrays consisting of two distinct floral forms, representing two *Clivia* species; plain orange *C. gardenii*-shaped models *versus* plain orange *C. miniata*-shaped models. Experimental arrays conducted at the MPNR site in October 2010. Observations (*n* = 25) recorded over five full days, and over 13 unique time blocks. Approaches; *n* = 20; brushes; *n* = 4, alights; *n* = 1.

Figure 8. Behavioural responses of butterfly species, across two sites, to model flower arrays consisting of two *C. miniata*-shaped choices (both orange with yellow target models); unscented (paraffin control) versus scent supplemented. Experimental arrays conducted at the MPNR site in 2010 and the UNR site in 2014. MPNR observations (*n* =185) recorded over 6 full days, and over 18 unique time blocks. Approaches; *n* = 145, brushes; *n* = 27, alights; *n* = 13. UNR observations (*n* =181) recorded over 4 full days, over 54 time blocks. Approaches; *n* = 147, brushes; *n* = 33, alights; *n* = 1.

Figure 9. Behavioural responses of butterfly species to two real flower choices, consisting of *C. miniata* versus *C. miniata* var. *citrina* inflorescences, at the UNR site over two consecutive seasons (2013 and 2014). 2013 observations (*n* = 169) recorded over 3 full days, and over 28 unique time blocks. Approaches; *n* = 92, brushes; *n* = 32, alights; *n* = 45. 2014 observations (*n* = 525) recorded over 5 full days, and over 52 unique time blocks. Approaches; *n* = 309; brushes; *n* = 64, alights; *n* = 152.

Figure 10. Behavioural responses of *P. dardanus cena* and *P. nireus lyaeus* to two real flower arrays consisting of *C. miniata* flowers in their normal upright orientation versus *C. miniata* flowers manipulated into a pendulous orientation. Experimental arrays conducted at the UNR site in the flowering season of 2014. Observations (*n* = 326) recorded over 5 full days, and over 53 unique time blocks. Approaches; *n* = 185, brushes; *n* = 103, alights; *n* = 38.

Figure 11. Behavioural responses of three butterfly species (*Belenois zochalia zochalia*, *P. dardanus cena* and *P. nireus lyaeus*) to two real flower arrays consisting of *C. miniata* flowers; unscented (paraffin control) versus scent supplemented. Experiments conducted in UNR in the flowering season of 2014. Observations (*n* = 271) recorded over 5 full days, and over 25 unique time blocks. Approaches; *n* = 186, brushes; *n* = 45, alights; *n* = 40.

Figure 12. Electroantennographic detection (EAD) responses of *Papilio* antennae to artificially blended *C. miniata* volatiles. MS – Mass spectrometer trace; EAD – eletroantennal trace (Scale: EAD trace). (A) Male *P. dardanus cena*. (B) Female *P. dardanus cena*. (C) Male *P. demodocus*.

Figure 1.

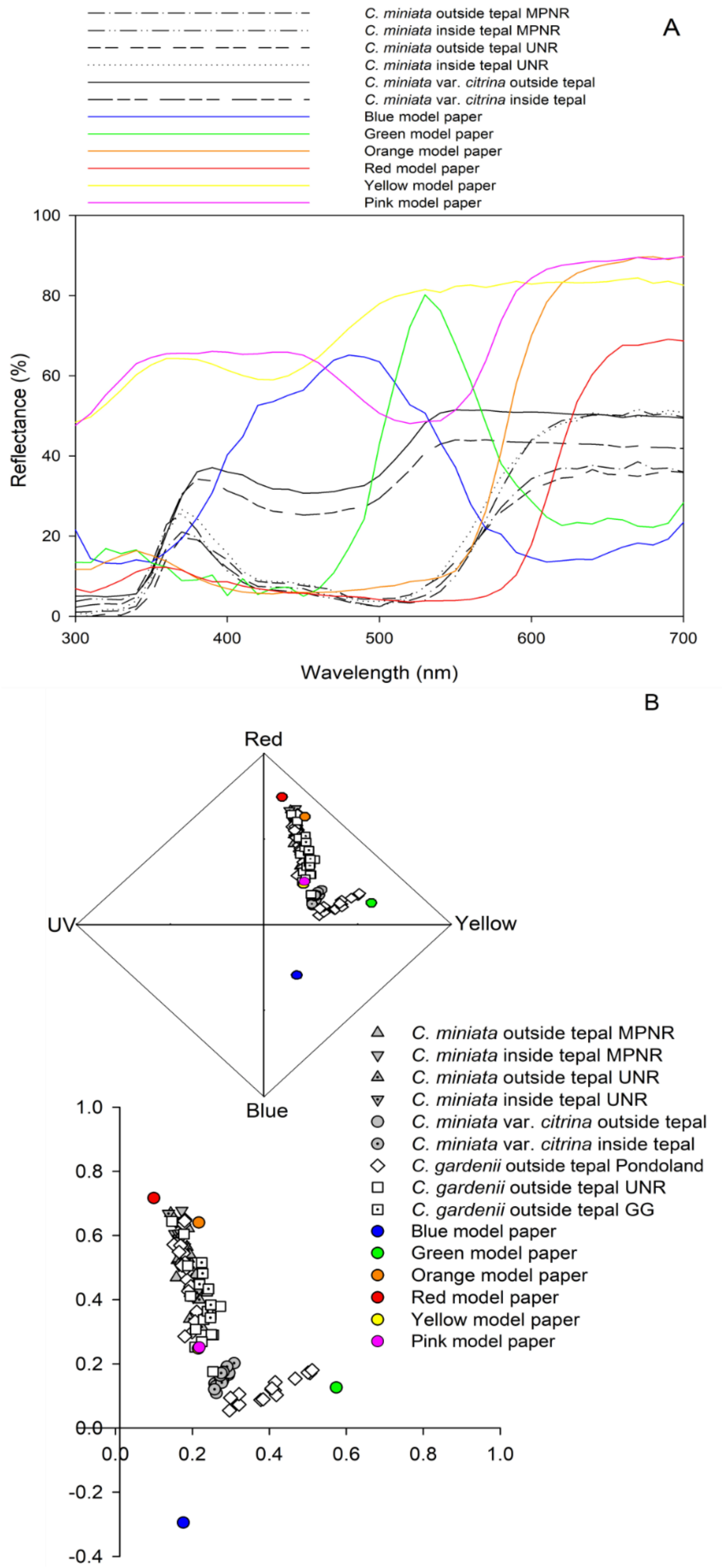

A
C. miniata outside tepal MPNR
C. miniata inside tepal MPNR
C. miniata outside tepal UNR
C. miniata inside tepal UNR
C. miniata var. citrina outside tepal
C. miniata var. citrina inside tepal
Blue model paper
Green model paper
Orange model paper
Red model paper
Yellow model paper
Pink model paper
Reflectance (%)
0
20
40
60
80
100
300
400
500
600
700
Wavelength (nm)
B
Red
UV
Yellow
Blue
C. miniata outside tepal MPNR
C. miniata inside tepal MPNR
C. miniata outside tepal UNR
C. miniata inside tepal UNR
C. miniata var. citrina outside tepal
C. miniata var. citrina inside tepal
C. gardenii outside tepal Pondoland
C. gardenii outside tepal UNR
C. gardenii outside tepal GG
Blue model paper
Green model paper
Orange model paper
Red model paper
Yellow model paper
Pink model paper
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0.0
0.2
0.4
0.6
0.8
1.0

Figure 2.

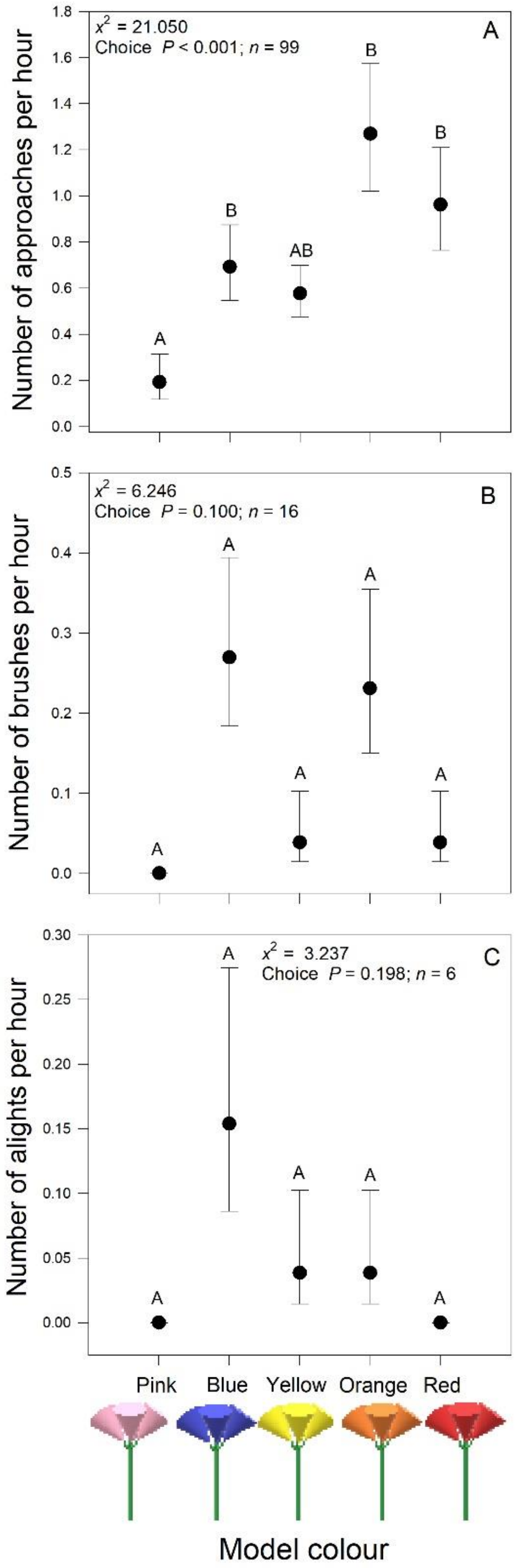
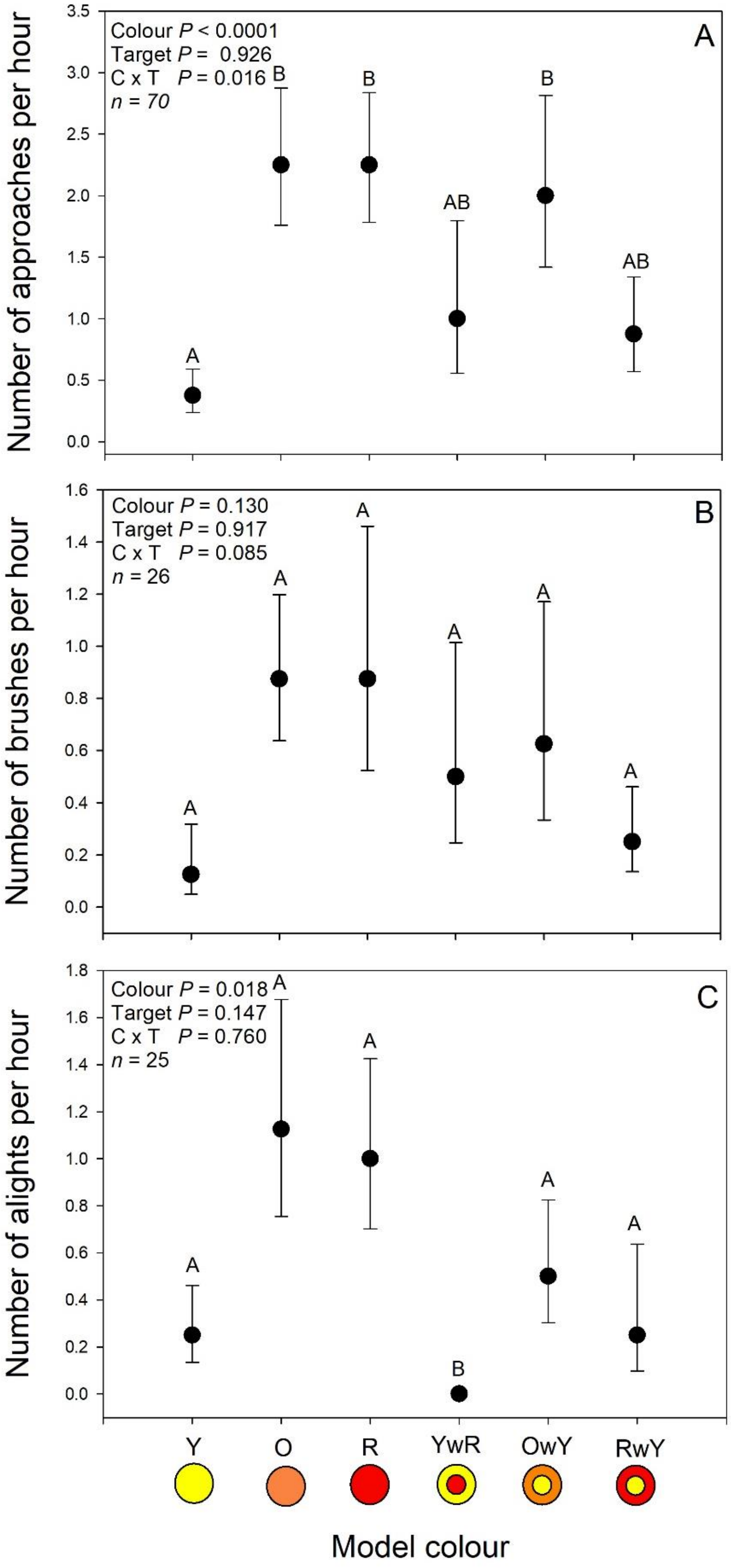

x^2 = 21.050
Choice P < 0.001; n = 99
A
Number of approaches per hour
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
AB
B
B
x^2 = 6.246
Choice P = 0.100; n = 16
B
Number of brushes per hour
0.5
0.4
0.3
0.2
0.1
0.0
A
A
A
A
A
x^2 = 3.237
Choice P = 0.198; n = 6
C
Number of alights per hour
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A
A
A
A
A
Pink
Blue
Yellow
Orange
Red
Model colour

Figure 3.



Number of approaches per hour
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Colour P < 0.0001
Target P = 0.926
C x T P = 0.016
n = 70
A
B
B
AB
B
AB
A
Number of brushes per hour
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Colour P = 0.130
Target P = 0.917
C x T P = 0.085
n = 26
B
A
A
A
A
A
A
Number of alights per hour
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Colour P = 0.018
Target P = 0.147
C x T P = 0.760
n = 25
C
A
A
A
B
A
A
Y
O
R
YwR
OwY
RwY
Model colour

Figure 4.

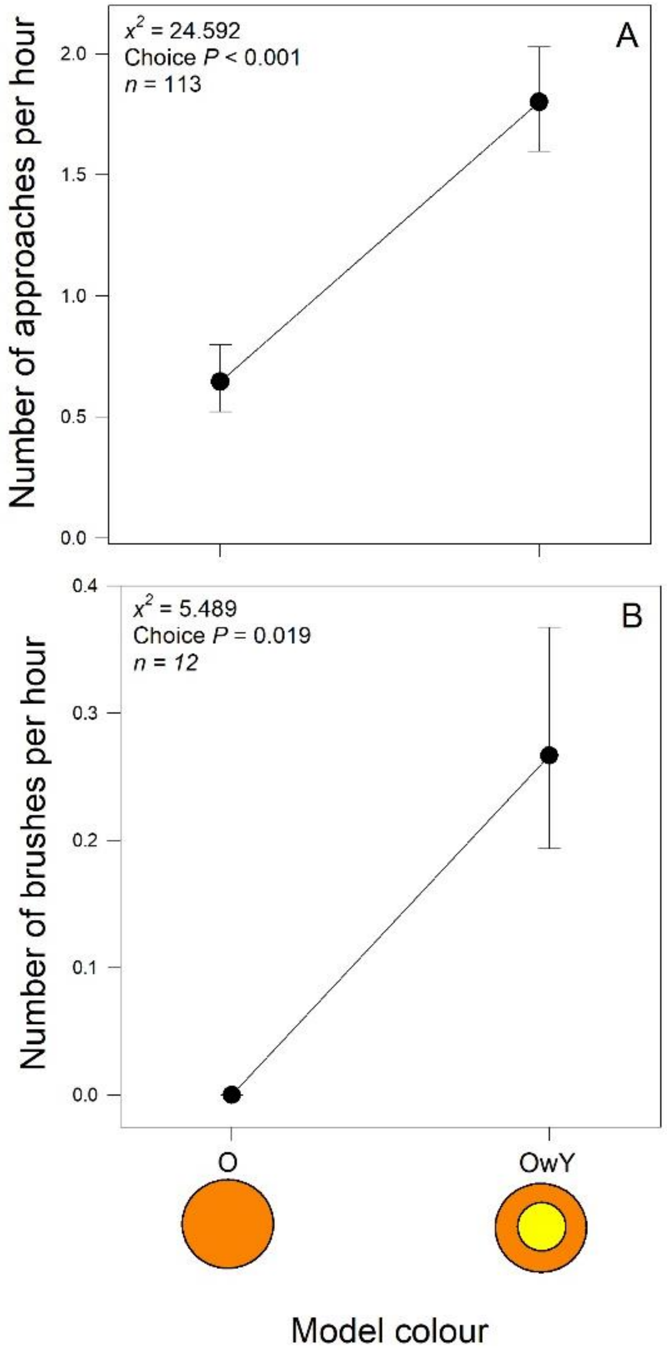

$x^2 = 24.592$
Choice $P < 0.001$
$n = 113$
A
Number of approaches per hour
0.0
0.5
1.0
1.5
2.0
$x^2 = 5.489$
Choice $P = 0.019$
$n = 12$
B
Number of brushes per hour
0.0
0.1
0.2
0.3
0.4
O
OwY
Model colour

Figure 5.

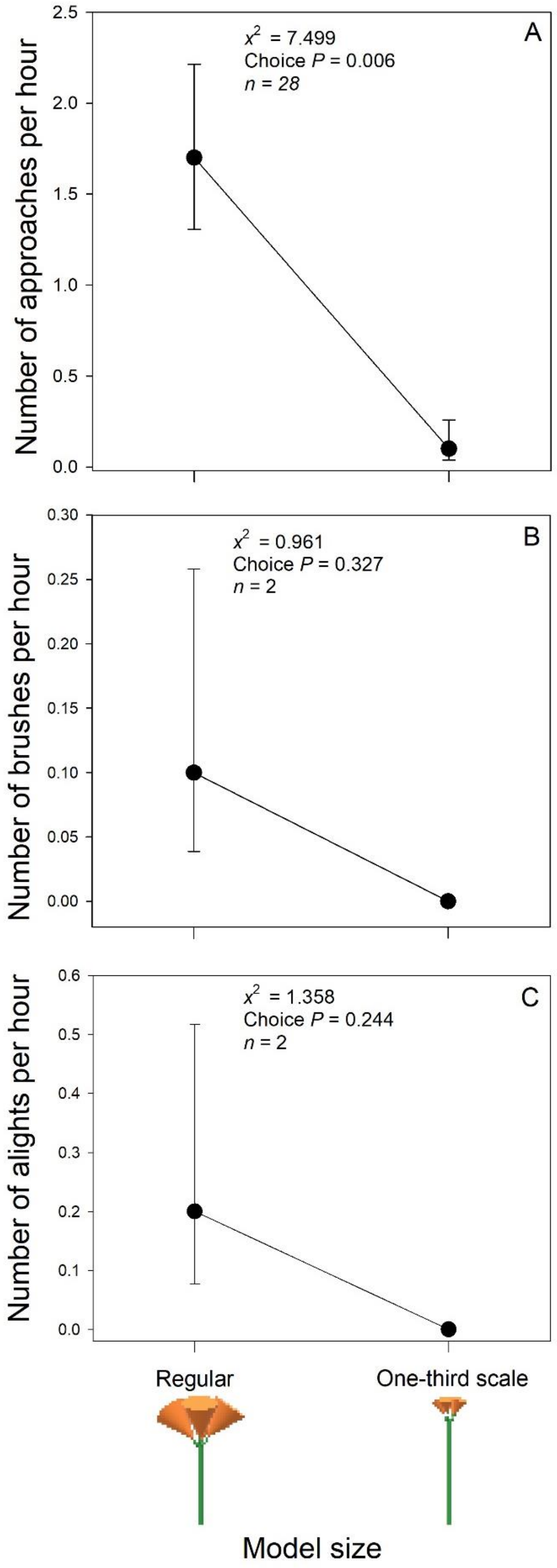
A
x^2 = 7.499
Choice P = 0.006
n = 28
Number of approaches per hour
0.0
0.5
1.0
1.5
2.0
2.5
B
x^2 = 0.961
Choice P = 0.327
n = 2
Number of brushes per hour
0.00
0.05
0.10
0.15
0.20
0.25
0.30
C
x^2 = 1.358
Choice P = 0.244
n = 2
Number of alights per hour
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Regular
One-third scale
Model size

Figure 6.

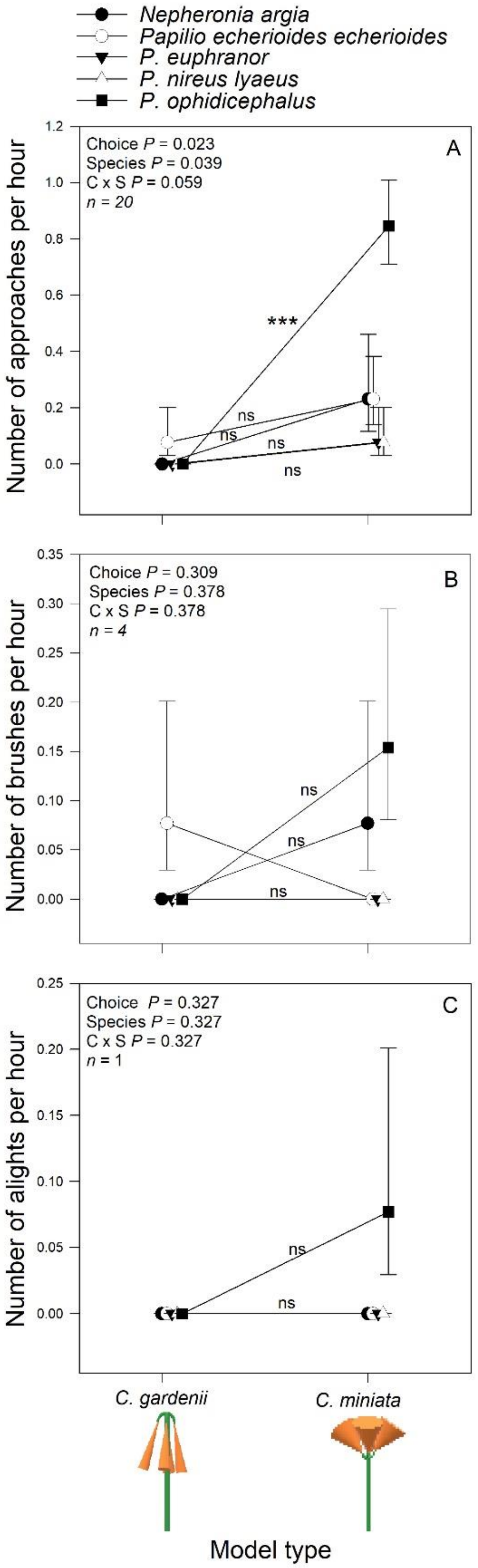
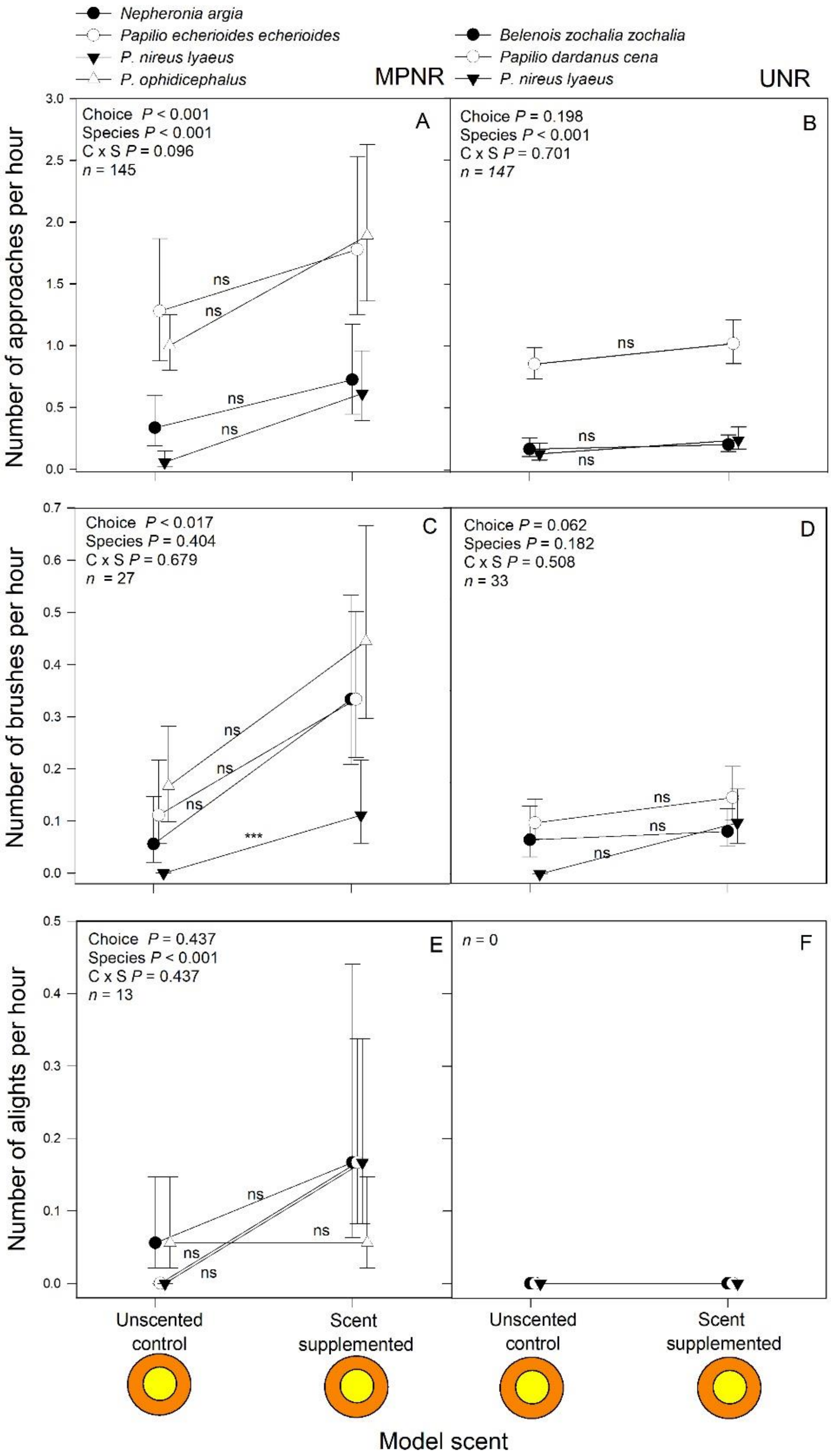

Nepheronia argia
Papilio echerioides echerioides
P. euphranor
P. nireus lyaeus
P. ophidicephalus
Number of approaches per hour
Choice P = 0.023
Species P = 0.039
C x S P = 0.059
n = 20
A
***
ns
ns
ns
ns
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Number of brushes per hour
Choice P = 0.309
Species P = 0.378
C x S P = 0.378
n = 4
B
ns
ns
ns
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Number of alights per hour
Choice P = 0.327
Species P = 0.327
C x S P = 0.327
n = 1
C
ns
ns
0.25
0.20
0.15
0.10
0.05
0.00
C. gardenii
C. miniata
Model type

Figure 7.



Nepheronia argia
Papilio echerioides echerioides
P. nireus lyaeus
P. ophidicephalus
MPNR
Belenois zochalia zochalia
Papilio dardanus cena
P. nireus lyaeus
UNR
Number of approaches per hour
Choice P < 0.001
Species P < 0.001
C x S P = 0.096
n = 145
A
Choice P = 0.198
Species P < 0.001
C x S P = 0.701
n = 147
B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ns
ns
ns
ns
ns
ns
ns
Number of brushes per hour
Choice P < 0.017
Species P = 0.404
C x S P = 0.679
n = 27
C
Choice P = 0.062
Species P = 0.182
C x S P = 0.508
n = 33
D
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ns
ns
ns
***
ns
ns
ns
Number of alights per hour
Choice P = 0.437
Species P < 0.001
C x S P = 0.437
n = 13
E
n = 0
F
0.5
0.4
0.3
0.2
0.1
0.0
ns
ns
ns
ns
Unscented control
Scent supplemented
Unscented control
Scent supplemented
Model scent

Figure 8.

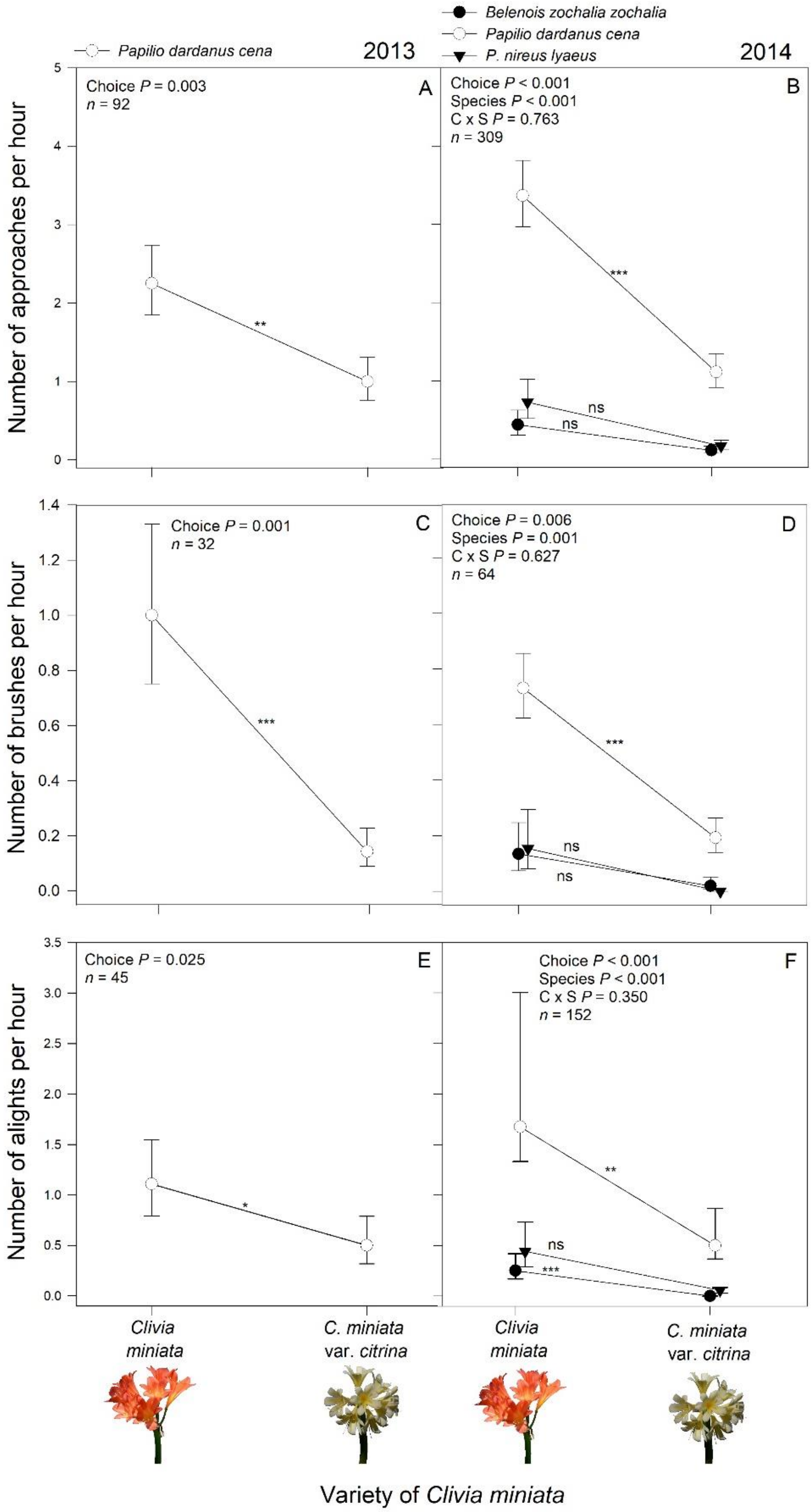

Papilio dardanus cena
2013
Belenois zochalia zochalia
Papilio dardanus cena
P. nireus lyaeus
2014
Number of approaches per hour
Choice P = 0.003
n = 92
A
**
Choice P < 0.001
Species P < 0.001
C x S P = 0.763
n = 309
B
***
ns
ns
0
1
2
3
4
5
Number of brushes per hour
Choice P = 0.001
n = 32
C
***
Choice P = 0.006
Species P = 0.001
C x S P = 0.627
n = 64
D
***
ns
ns
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Number of alights per hour
Choice P = 0.025
n = 45
E
*
Choice P < 0.001
Species P < 0.001
C x S P = 0.350
n = 152
F
**
ns
***
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Clivia miniata
C. miniata var. citrina
Clivia miniata
C. miniata var. citrina
Variety of Clivia miniata

Figure 9.

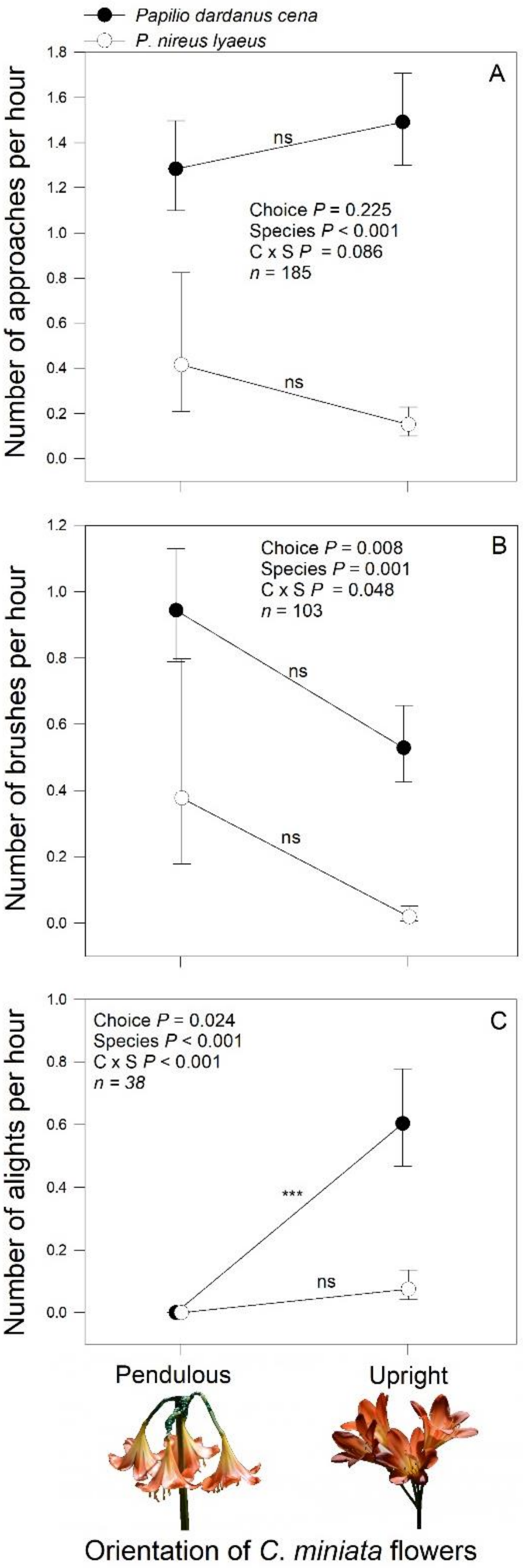

Papilio dardanus cena
P. nireus lyaeus
A
Number of approaches per hour
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
Choice P = 0.225
Species P < 0.001
C x S P = 0.086
n = 185
ns
B
Number of brushes per hour
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Choice P = 0.008
Species P = 0.001
C x S P = 0.048
n = 103
ns
ns
C
Number of alights per hour
1.0
0.8
0.6
0.4
0.2
0.0
Choice P = 0.024
Species P < 0.001
C x S P < 0.001
n = 38
***
ns
Pendulous
Upright
Orientation of C. miniata flowers

Figure 10.

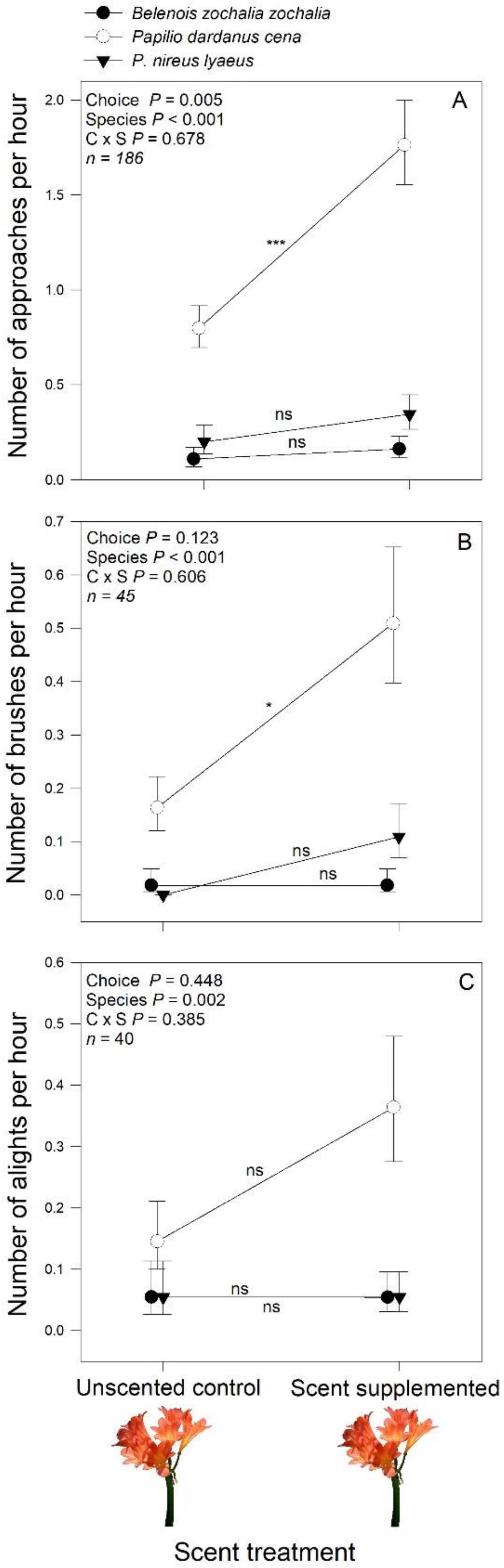

Belenois zochalia zochalia
Papilio dardanus cena
P. nireus lyaeus
Number of approaches per hour
2.0
1.5
1.0
0.5
0.0
Choice P = 0.005
Species P < 0.001
C x S P = 0.678
n = 186
A
***
ns
ns
Number of brushes per hour
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Choice P = 0.123
Species P < 0.001
C x S P = 0.606
n = 45
B
*
ns
ns
Number of alights per hour
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Choice P = 0.448
Species P = 0.002
C x S P = 0.385
n = 40
C
ns
ns
ns
Unscented control
Scent supplemented
Scent treatment

Figure 11.

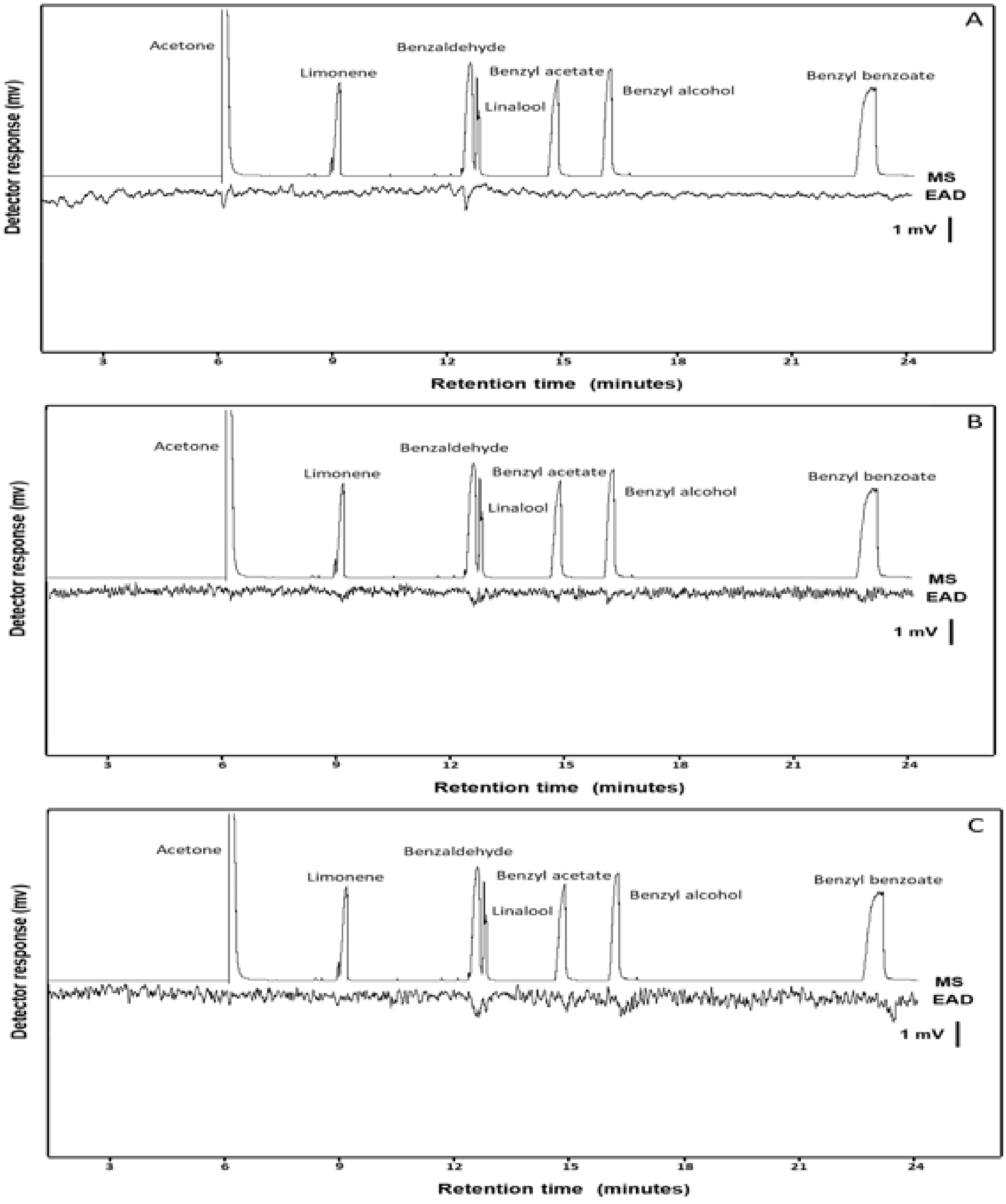

A
Acetone
Limonene
Benzaldehyde
Benzyl acetate
Linalool
Benzyl alcohol
Benzyl benzoate
MS
EAD
1 mV
Detector response (mv)
3
6
9
12
15
18
21
24
Retention time (minutes)
B
Acetone
Limonene
Benzaldehyde
Benzyl acetate
Linalool
Benzyl alcohol
Benzyl benzoate
MS
EAD
1 mV
Detector response (mv)
3
6
9
12
15
18
21
24
Retention time (minutes)
C
Acetone
Limonene
Benzaldehyde
Benzyl acetate
Linalool
Benzyl alcohol
Benzyl benzoate
MS
EAD
1 mV
Detector response (mv)
3
6
9
12
15
18
21
24
Retention time (minutes)

Figure 12.

APPENDIX 2

Appendix S1. Butterfly species visiting flower arrays at various sites and statistical details for each bioassay.

Bioassay and butterfly species visiting each array	**Figure**	**Site and year**	**Model type**	**Link function**	**Dependent variable**	**Model source**	**Wald χ2**	**df**	***P***
Five colour model	3	UNR	Negative Binomial	Log	Approach (*n* = 99)	Choice	21.050	4	< 0.001
Papilio dardanus cena		2013				Overall test results	25.782	4	< 0.001
					Brush (*n* = 16)	Choice	6.246	3	0.100
						Overall test results	11.824	4	0.019
					Alight (*n* = 6)	Choice	3.237	2	0.198
						Overall test results	4.450	3	0.217
Colour pattern model	4	MPNR	Negative Binomial	Log	Approach (*n* = 70)	Colour*Target	8.235	2	0.016
Nepheronia argia		2010				Colour	39.020	2	< 0.001
Papilio euphranor						Target	0.009	1	0.926
Papilio dardanus cena						Overall test results	95.668	5	< 0.001
Papilio ophidicephalus					Brush (*n* = 26)	Colour*Target	4.927	2	0.085
Papilio echerioides echerioides						Colour	4.078	2	0.130
						Target	0.011	1	0.917
						Overall test results	206.093	5	< 0.001
					Alight (*n* = 25)	Colour*Target	0.548	2	0.760
						Colour	8.056	2	0.018
						Target	2.107	1	0.147
						Overall test results	10.335	4	0.035
Orange target, no-target model	5	UNR	Negative Binomial	Log	Approach (*n* = 113)	Choice	24.592	1	< 0.001
Papilio dardanus cena		2013				Overall test results	24.592	1	< 0.001

					Brush (*n* = 12)	Choice	5.489	1	0.019
						Overall test results	5.489	1	0.019
			Binomial	Logit	First choice	Intercept	25.304	1	< 0.001
					n = 113; Mean = 0.7417; Lower SE = 0.6995; Upper SE = 0.7799; 95% Wald confidence interval: Lower 0.644; Upper 1.467 Response = orange with target; Reference category = orange				
Regular vs. reduced size model	6	MPNR	Negative Binomial	Log	Approach (*n* = 28)	Choice	7.499	1	0.006
Papilio ophidicephalus		2010				Overall test results	7.499	1	0.006
					Brush (*n* = 2)	Choice	0.961	1	0.327
						Overall test results	0.961	1	0.327
					Alight (*n* = 2)	Choice	1.358	1	0.244
						Overall test results	1.358	1	0.244
			Binomial	Logit	First choice	Intercept	13.653	1	< 0.001
					n = 28; Mean = 0.9307; Lower SE = 0.8692; Upper SE = 0.9644; 95% Wald confidence interval: Lower 1.220; Upper 3.975 Response = regular size; Reference category = 1/3 size				
***C. miniata* vs. *C. gardenii* model**	7	MPNR	Negative Binomial	Log	Approach (*n* = 20)	Choice*Species	9.086	4	0.059
Nepheronia argia		2010				Choice	5.203	2	0.023
Papilio echerioides echerioides						Species	10.114	4	0.039
Papilio euphranor						Overall test results	184.506	6	< 0.001
Papilio nireus lyaeus					Brush (*n* = 4)	Choice*Species	1.947	2	0.378
Papilio ophidicephalus						Choice	1.035	1	0.309

						Species	1.947	2	0.378
						Overall test results	2.364	2	0.307
					Alight (*n* = 1)	Choice*Species	0.961	1	0.327
						Choice	0.961	1	0.327
						Species	0.961	1	0.327
						Overall test results	1.083	1	0.298
			Binomial	Logit	First choice	Intercept	8.988	1	0.003
					n = 20; Mean = 0.9489; Lower SE = 0.8751; Upper SE = 0.9801; 95% Wald confidence interval: Lower 1.012; Upper 4.832 Response = *C. miniata*; Reference category = *C. gardenii*				
Unscented vs. scented model	8	MPNR	Negative Binomial	Log	Approach (*n* = 145)	Choice*Species	6.356	3	0.096
Nepheronia argia		2010				Choice	19.597	1	< 0.001
Papilio echerioides echerioides						Species	24.676	3	< 0.001
Papilio nireus lyaeus						Overall test results	70.920	7	< 0.001
Papilio ophidicephalus					Brush (*n* = 27)	Choice*Species	0.773	2	0.679
						Choice	5.686	1	0.017
						Species	2.920	3	0.404
						Overall test results	7264.896	6	< 0.001
					Alight (*n* = 13)	Choice*Species	0.603	1	0.437
						Choice	0.603	1	0.437
						Species	66.080	3	< 0.001
						Overall test results	5.143	3	0.273
			Binomial	Logit	First choice	Intercept	18.510	1	< 0.001

					n = 145; Mean = 0.6557; Lower SE = 0.6210; Upper SE = 0.6887; 95% Wald confidence interval: Lower 0.350; Upper 0.937 Response = unscented; Reference category = scented				
Unscented vs. scented model	8	UNR	Negative Binomial	Log	Approach (n = 147)	Choice*Species	0.711	2	0.701
Belenois zochalia zochalia		2014				Choice	1.658	1	0.198
Papilio dardanus cena						Species	58.725	2	< 0.001
Papilio nireus lyaeus						Overall test results	65.448	5	< 0.001
					Brush (n = 33)	Choice*Species	1.353	2	0.508
						Choice	3.494	1	0.062
						Species	3.411	2	0.182
						Overall test results	14.179	5	0.015
					Alight (n = 0)	Choice*Species	0 visits		
						Choice			
						Species			
						Overall test results			
			Binomial	Logit	First choice	Intercept	2.667	1	0.102
					n = 147; Mean = 0.5977; Lower SE = 0.5382; Upper SE = 0.6545; 95% Wald confidence interval: Lower - 0.079; Upper 0.872 Response = unscented; Reference category = scented				
Real *C. miniata* vs. *C. miniata* var. *citrina*	9	UNR	Negative Binomial	Log	Approach (n = 92)	Choice	8.881	1	0.003
Papilio dardanus cena		2013				Overall test results	8.881	1	0.003
					Brush (n = 32)	Choice	11.418	1	0.001
						Overall test results	11.418	1	0.001

					Alight (*n* = 45)	Choice	4.999	1	0.025
						Overall test results	4.999	1	0.025
			Binomial	Logit	First choice	Intercept	9.306	1	0.002
					n = 92; Mean = 0.6833; Lower SE = 0.6264; Upper SE = 0.7352; 95% Wald confidence interval: Lower 0.275; Upper 1.263 Response = *C. miniata*; Reference category = *C. miniata* var. *citrina*				
Real *C. miniata* vs. *C. miniata* var. *citrina* *Belenois zochalia zochalia* *Papilio dardanus cena* *Papilio nireus lyaeus*	9	UNR 2014	Negative Binomial	Log	Approach (*n* = 309)	Choice*Species	0.541	2	0.763
						Choice	33.597	1	< 0.001
						Species	66.396	2	< 0.001
						Overall test results	86.815	5	< 0.001
					Brush (*n* = 64)	Choice*Species	0.237	1	0.627
						Choice	7.456	1	0.006
						Species	13.869	2	0.001
						Overall test results	57.450	5	< 0.001
					Alight (*n* = 152)	Choice*Species	0.873	1	0.350
						Choice	13.059	1	< 0.001
						Species	22.656	2	< 0.001
						Overall test results	14704.237	4	< 0.001
			Binomial	Logit	First choice	Intercept	41.515	1	< 0.001
					n = 309; Mean = 0.7667; Lower SE = 0.7320; Upper SE = 0.7982; 95% Wald confidence interval: Lower 0.828; Upper 1.552 Response = *C. miniata*; Reference category = *C. miniata* var. *citrina*				

Real upright vs. pendulous oriented *C. miniata* *Papilio dardanus cena* *Papilio nireus lyaeus*	10	UNR 2014	Negative Binomial	Log	Approach (*n* = 185)	Choice*Species	2.951	1	0.086
						Choice	1.474	1	0.255
						Species	17.661	1	< 0.001
						Overall test results	60.994	3	< 0.001
					Brush (*n* = 103)	Choice*Species	3.903	1	0.048
						Choice	7.060	1	0.008
						Species	11.604	1	0.001
						Overall test results	43.665	3	< 0.001
					Alight (*n* = 38)	Choice*Species	15.958	1	< 0.001
						Choice	5.067	1	0.024
						Species	15.958	1	< 0.001
						Overall test results	14.806	2	0.001
			Binomial	Logit	First choice	Intercept	0.414	1	0.520
					n = 185; Mean = 0.5282; Lower SE = 0.4845; Upper SE = 0.5715; 95% Wald confidence interval: Lower -0.230; Upper 0.455 Response = upright orientation; Reference category = pendulous orientation				
Real unscented vs. scent supplemented *C. miniata* *Belenois zochalia zochalia* *Papilio dardanus cena* *Papilio nireus lyaeus*	11	UNR 2014	Negative Binomial	Log	Approach (*n* = 186)	Choice*Species	0.779	2	0.678
						Choice	7.883	1	0.005
						Species	58.406	2	< 0.001
						Overall test results	98.973	5	< 0.001
					Brush (*n* = 45)	Choice*Species	1.003	1	0.606
						Choice	2.379	1	0.123
						Species	22.865	2	< 0.001
						Overall test results	21.304	5	0.001

		Alight (*n* = 40)	Choice*Species	1.910	2	0.385
			Choice	0.574	1	0.448
			Species	12.076	2	0.002
			Overall test results	25.631	5	< 0.001
Binomial	Logit	First choice	Intercept	19.819	1	< 0.001

n = 186; Mean = 0.6691; Lower SE = 0.6332; Upper SE = 0.7031;
95% Wald confidence interval: Lower 0.394; Upper 1.014
Response = scent supplemented; Reference category = unscented control

CHAPTER 5

SPIT IT OUT: MONKEYS DISPERSE THE UNORTHODOX AND TOXIC SEEDS OF *CLIVIA MINIATA* (AMARYLLIDACEAE)

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INSIGHTS

WILEY bioTROPICA ASSOCIATION FOR TROPICAL BIOLOGY AND CONSERVATION

Spit it out: Monkeys disperse the unorthodox and toxic seeds of *Clivia miniata* (Amaryllidaceae)

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Abstract

Seeds of many Amaryllidaceae are unorthodox (recalcitrant) and toxic, and cannot survive ingestion, yet are packaged in brightly colored fruits suggestive of zoochory. Seed dispersal and germination of the understory amaryllid, *Clivia miniata*, were investigated in KwaZulu-Natal, South Africa. Motion-activated cameras revealed that samango monkeys (*Cercopithecus mitis labiatus*) are the primary disperser of *C. miniata* seeds. They eat the mesocarp and, to a lesser extent, the exocarp, and spit the large (13 mm diameter) seeds whole and cleaned onto the forest floor. Most seeds were dispersed farther than 1 m from the parent. Experimental removal of the fruit pulp had a small positive effect on the rate of seed germination, but did not affect subsequent seedling growth rates. The main advantages of monkey dispersal of *Clivia* seeds appear to be short-distance dispersal away from the dense foliage of clumped parent plants and occasional long-distance dispersal through cheek-pouching behavior.

KEYWORDS

Camera traps, *Cercopithecus mitis labiatus*, Coastal-lowland Forest, germination, guenon, Mistbelt Forest, seed distribution, South Africa

1 | INTRODUCTION

Seed dispersers of most plant species and their effect on seedling establishment remain unknown—a problem that is particularly acute for rare species in remote localities. Motion-activated cameras offer new opportunities to document interactions between plants and animal seed dispersers (Midgley, White, Johnson & Bronner, 2015; Nyiramana, Mendoza, Kaplin & Forget, 2011; Seufert, Linden & Fischer, 2010).

Diversification of Amaryllidaceae is closely associated with the evolution of novel seed and fruit traits (Snijman & Linder, 1996); however, information on zoochory in the family has been almost completely absent. The seeds of many species in the tribe Haemantheae have seeds that are both toxic (Crouch, Mulholland, Pohl & Ndlovu, 2003; Viladomat, Bastida, Codina, Nair & Campbell, 1997) and recalcitrant (i.e., "unorthodox"; Meerow & Snijman, 1998). They are thus unlikely to be ingested by animals, and, any that are, are unlikely to survive gut passage because they lack a protective seed coat. Any dispersal and subsequent germination of such seeds is instead likely to be the by-product of animals removing the edible pulp that surrounds them. "De-pulping" may have the additional benefits of enhancing germination rates through the removal of germination inhibitors (Evenari, 1949; Levey, 1987), reducing the potential for seed mortality from fungal and bacterial pathogens (Levi & Peres, 2013; Moore, 2001; Witmer & Cheke, 1991) and subsequent spitting or defecation offer seed movement from the parent (Schupp, 1993).

Clivia miniata (Lindl.) Bosse (Amaryllidaceae: Haemantheae) is one of the world's most widely cultivated ornamental plants (Koopowitz, 2002), yet its seed dispersal system remains undescribed. The berries of this species typically have a colorful endocarp when ripe, and the mesocarp is fleshy and sweet-tasting (I. Kiepiel, personal observation), suggesting animal-mediated dispersal. In addition, *C. miniata* seeds are toxic (Nelson, Shih & Balick, 2007; Viladomat et al., 1997; van Wyk, van Oudtshoorn & Gericke, 2009) and therefore would need to be separated from the fruit to prevent ingestion. We therefore predict that they are dispersed by primates, which can readily manipulate the fruits with their hands or mouths. We also predict that the de-pulping of seeds by primates would increase their rate of germination and hence seedling establishment.

2 | METHODS

2.1 | Study species and sites

Clivia miniata inhabits understory coastal and Afromontane forests in the eastern part of South Africa (Swanevelder & Fisher, 2009). The large orange flowers are pollinated by swallowtail butterflies and fruits ripen slowly over ca. 12 months (Kiepiel & Johnson, 2014b). The mean (±SD) percentage of flowers that set fruit in a previous study was 25.30 ± 0.436 and each fruit contained 2.49 ± 1.609 seeds (Kiepiel & Johnson, 2014a). Typical interplant distances vary from 10 cm to 30 cm. Fruits are presented 69 ± 8.12 cm from the ground. The mass of fruits is 2.9 ± 1.52 g, and they are 21.0 ± 4.7 mm long and 16.6 ± 3.11 mm wide (*n* = 86). Seeds are 14.7 ± 1.57 mm long and 13.3 ± 3.11 mm wide (*n* = 155). Research was conducted at three forest sites, Umtamvuna (UNR), Mbona (MPNR), and Bushwillow (BW) from May to September 2017 (for details see Table S1).

2.2 | Camera traps and observations of fruit removal

We used camera traps (Bushnell® 14 Mp NatureView Cam HD) to record animals interacting with *C. miniata* fruits during the Austral winter and spring. Six cameras were used at each of UNR and MPNR, and three cameras were deployed at BW. The number of fruits in the field of view of each camera was recorded before and after the experiment (see Supplementary methods for detail and camera settings). Dispersal was monitored for a mean of 100 days per camera (1502 camera days in total) across the three forest sites. From videos, we recorded, where possible, the number of fruits removed per plant, the proportion of fruit removed per plant by each monkey, and the fruit-handling time (from when a fruit was picked to when the last seed or piece of exocarp was discarded). Dispersal distance for discarded seeds was estimated using plant parts and the animal as a scale, and if seeds were taken beyond the field of view, a minimum distance was recorded. We scored fruits as ripe (red or mostly red), semiripe (mostly green), and unripe (entirely green). We did not record any removal of green fruits younger than eight months. However, entirely green fruits that have matured for eight months contain viable seeds that are able to germinate (I. Kiepiel, unpublished data). Fruit picked from a single plant by an individual monkey was usually at the same maturation stage, thus preventing us from directly establishing preferences for ripe fruit.

2.3 | Fruit distribution surveys and secondary dispersal tests

To establish distances of seed dispersal and potential predation beyond the range of cameras, we conducted bi-weekly surveys within each population and in zones that extended roughly 75 m beyond the population into the surrounding forest. We conducted transects from the center of each population, and the distances between fruits and seeds on the forest floor and the base of the closest possible parent were recorded.

To identify possible secondary dispersal agents or seed predation, we placed a cache of 25 ripe fruits and 20 peeled, cleaned, ripe seeds on a flat rock in the view of a motion camera at UNR over a period of 39 days and repeated this design with five fruits and five seeds at each of MPNR and BW.

2.4 | Seed germination and seedling growth

We investigated the effects of exocarp and mesocarp removal on seed germination and seedling growth using ripe fruits from MPNR. Fruits were collected from 34 plants in pairs and were randomly assigned to either an unpeeled or a peeled treatment. Fruits were weighed to 0.01 g using a field portable scale (ACCULAB PP2060D). Fruits were peeled (de-pulped) by hand to remove all endocarp and mesocarp. The seeds from a single peeled fruit (for peeled treatment: *n* = 71 seeds; mean ± SE of 1.73 ± 0.152 seeds per fruit) or an intact unpeeled fruit (for unpeeled treatment: *n* = 73 seeds; mean ± SE of 1.78 ± 0.162 seeds per fruit) were then planted in a 5 × 5 cm plug in a seed-tray (six plugs per tray) filled with matured and blended organic soil comprised largely of compost. Pairs from each seed family were randomly allocated to these trays which were placed in a greenhouse under 50% shade and watered *ad libitum*. Germination (radical emergence) and emergence of the first leaf were scored weekly until most seeds had either germinated or died. The lengths of the longest leaf on each plant were measured after 40 weeks.

2.5 | Statistical analysis

We used generalized linear models (GLMs) implemented in SPSS version 25 (IBM Corp.) that incorporated a Poisson distribution and log link function for analyses of count data, binomial distribution and logit link function for analyses of proportion data, gamma distribution and log link function for right-skewed data (e.g., handling time per fruit and dispersal distance of seeds), and Gaussian distribution and identity link function for morphological measurements (see Table S2). Analyses of field data included site as a fixed factor, whereas those of greenhouse data included cleaning treatment as a fixed factor. The analyses of leaf length and germination success included fruit mass and number of seeds as covariates. Analyses of the greenhouse experiment used generalized estimating equations (GEEs) to account for the paired application of treatments to individual plants, and within-plant variation was modeled with an exchangeable correlation matrix. Significance tests for GEE analyses

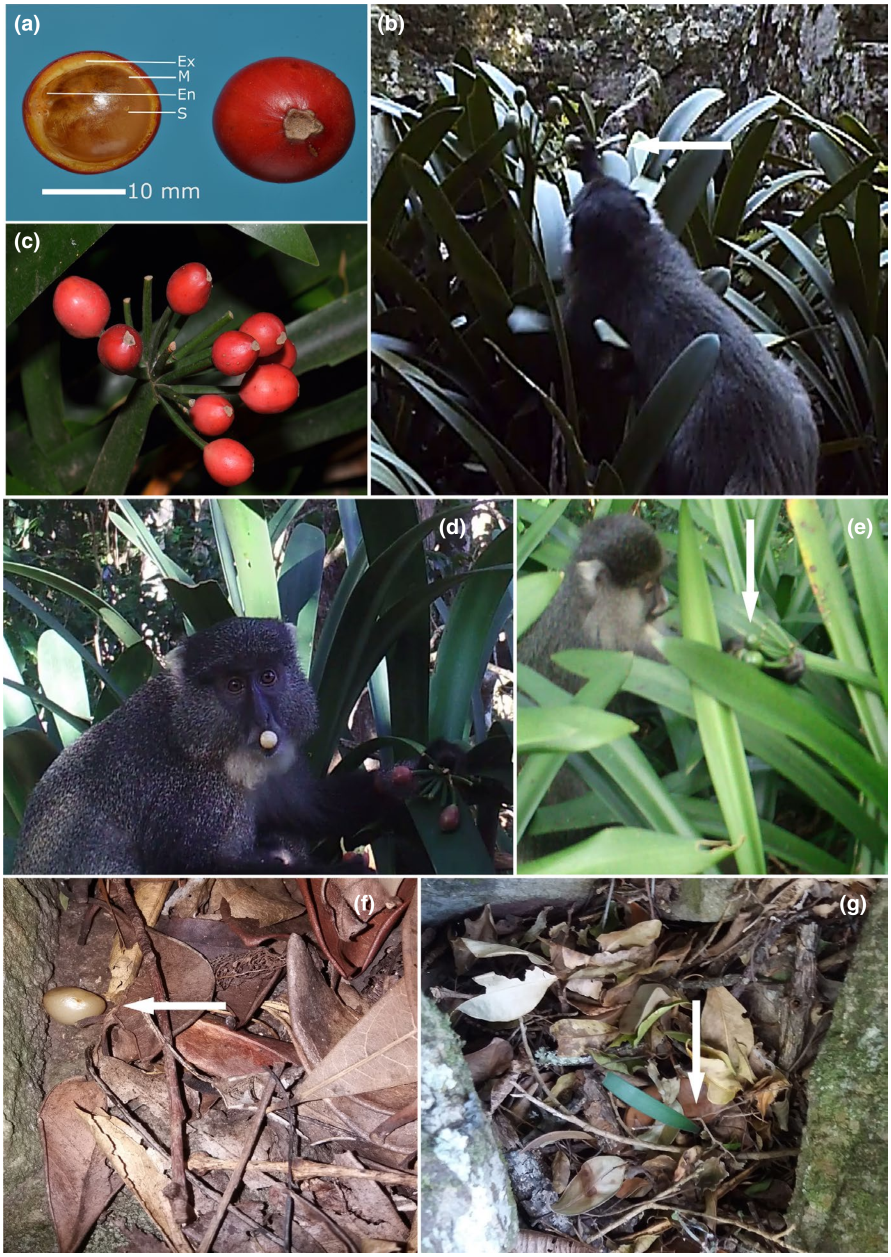

FIGURE 1 Development and dispersal of *Clivia miniata* seeds, illustrated by, (a) dissected *C. miniata* fruit (left) showing, En; endocarp, M; mesocarp, Ex; exocarp, S; seed and an entire *C. miniata* fruit (right) illustrating the remnants of the perianth from the UNR, (b) a subadult male *Cercopithecus mitis labiatus* picking unripe green fruit in UNR, (c) a 10-month-old peduncle with ripe fruits in UNR, (d) an adult female *C. mitis labiatus* with infant (barely visible), in UNR exhibiting typical spitting behavior—discarding peeled and undamaged seed, (e) a juvenile male *C. mitis labiatus* picking unripe green fruit while holding the peduncle in MPNR, (f) discarded processed seed on rock among leaf litter in the UNR, unlikely to germinate, and (g) a seedling established in leaf litter and humus collected between rocks in the UNR

involved Wald statistics. We also assessed the joint effects of peeling and germination time (weeks) on the cumulative proportion of germinated seeds using nonlinear sigmoidal model fitting (nlmixed procedure of SAS/STAT 14.2: SAS Institute Inc., 2016) as this provided the best fit to the data (see Pearson, Burslem, Mullins & Dalling, 2003; see Table S3).

3 | RESULTS

3.1 | Fruit removal

Camera traps (19 videos from 13 plants) revealed 36 cases (23 from UNR and 13 from MPNR) of *C. miniata* fruits (Figure 1a) being taken by samango monkeys (*Cercopithecus mitis labiatus*; Figure 1b,c,d; Supplementary video link in Video S1). No seed dispersal events were recorded from BW, and no fruits were removed from plants at this site. At MPNR, most fruit removal occurred in the morning around 0900 h, whereas at UNR removal during afternoon was most common (Figure S1). A single fruit was removed by a juvenile male *Tragelaphus sylvaticus* antelope (southern bushbuck) at MPNR.

Male, female, subadult, and juvenile samango monkeys were recorded walking on all fours through *C. miniata* stands, and picking fruits by hand (Figure 1b,c,d). Monkeys consumed both ripe and unripe fruit (Table 1). Monkeys ate the fleshy mesocarp and, to a lesser degree, the exocarp of *C. miniata* berries. Seeds were spat out clean and undamaged (Figure 1c; Video S1). No significant differences were observed in fruit removal or handling time between MPNR and UNR sites (Table 1). Although the proportion of seeds spat out could not be determined (as monkeys carried many fruits off camera; Table 1), eight seeds were videoed being spat out on camera (see Figure 1c; Video

TABLE 1 Handling of *Clivia miniata* fruit by samango monkeys (*Cercopithecus mitis labiatus*) at two forest sites (UNR and MPNR)

	UNR			MPNR					
	Mean	SE	*n*	Mean	SE	*n*	χ^2	*df*	*p*
Number of fruits removed per plant	1.77	0.369	13	2.17	0.601	6	0.318	1	.573
Prop. of fruit removed per plant	0.49	0.073	13	0.62	0.106	6	1.016	1	.313
Handling time per fruit (s)	13.91	1.837	19	19.05	1.762	11	4.083	1	.430
Estimated dispersal distance (cm)	63.68	11.094	19	66.92	14.095	13	0.033	1	.857
Prop. of seeds in mouth spat on camera	0.26	0.092	23	0.08	0.074	13	2.444	1	.118
Prop. of fruits picked that were ripe	0.39	0.102	23	0	0	13	14.786	1	<.001

S1). We observed cheek-pouching three times at the UNR site and twice at the MPNR site.

3.2 | Fruit distribution surveys and secondary dispersal

We found *C. miniata* seeds mostly less than 3 m from prospective parent plants (Figure 2). We found no signs of animal or fungal damage to seeds. Whole fruits found <1 m from prospective parents had likely fallen from parent plants when ripe, and the four cases of exocarp found without seeds were probably a result of them being discarded by samango monkeys, as this behavior was also seen on the videos (Video S1).

Seeds and ripe fruit placed in caches on the ground were not removed, even after several weeks. An olive thrush (*Turdus olivaceus*) and a ring-necked dove (*Streptopelia capicola*) were seen briefly pecking at ripe red fruit on the ground without removing them.

3.3 | Seed germination

Seeds freed from the mesocarp germinated faster and more frequently than those within intact fruits (Figure 3). The cumulative proportion of germinated seeds reached 50% of its asymptotic value within 7.4 weeks (SE = 0.35) for peeled seeds, compared to 8.8 ± 0.40 weeks for seeds from intact fruits (LR = 5.35, 1 *df*, *p* = .02). A higher proportion of peeled seeds ultimately germinated (asymptote: peeled, 0.85 ± 0.013; unpeeled, 0.76 ± 0.016: LR = 17.3, 1 *df*, *p* < .001). The mean proportion of seeds germinating when accounting for within-plant variation was 0.91 (0.846 lower SE, 0.943 upper SE) for peeled seeds versus 0.83 for unpeeled seed (0.778 lower SE, 0.866 upper SE; χ^2 = 1.266; *p* = .261). In this analysis, germination varied positively with fruit mass (β = 0.579; Wald χ^2 = 10.003; *df* = 1, *p* = .002) and negatively with seed number (β = −1.039; Wald χ^2 = 5.313; *df* = 1, *p* = .021). Leaf length of seedlings after 30 weeks similarly varied positively with fruit mass (β = 14.692; Wald χ^2 = 17.019; *df* = 1, *p* < .001) and negatively with the number of seeds per fruit (β = -4.459; Wald χ^2 = 4.051; *df* = 1, *p* = .044), but did not differ significantly between peeled and unpeeled treatments after 30 weeks (peeled: 111.6 mm ± 6.56; unpeeled: 112.6 mm ± 7.08; χ^2 = 0.012; *df* = 1, *p* = .912).

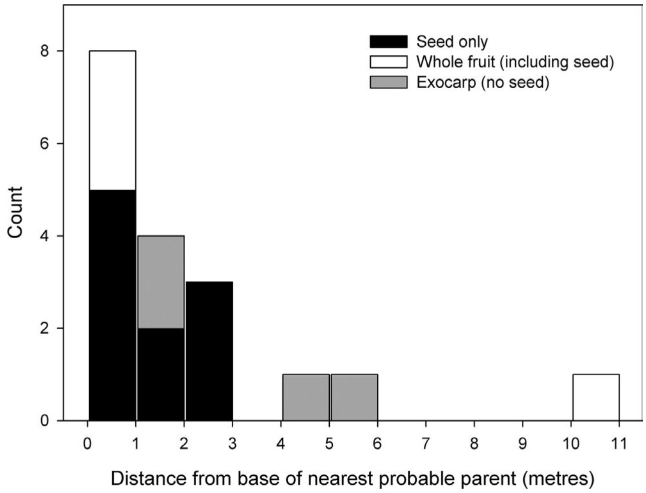

FIGURE 2 Distributions of the distances of *Clivia miniata* seeds and fruit from the nearest possible parent, based on transects at UNR

4 | DISCUSSION

Our results suggest that samango monkeys are the primary disperser of *C. miniata* seeds. *Cercopithecus* monkeys are significant seed dispersers in the Afrotropics (Garber & Lambert, 1998; Lambert, 2010; Lambert & Garber, 1998; Lawes, Henzi & Perrin, 1990; Linden, Linden, Fischer & Linsenmair, 2015; Seufert et al., 2010). Vervet monkeys (*Chlorocebus pygerythrus*) were not recorded in this study and tend to occupy more open habitats, but we cannot exclude the possibility that they also play a role in dispersal of *Clivia* fruits.

Clivia miniata seeds conform to the African "bird-monkey syndrome" - characterized by brightly colored berries and drupes and contrasts with the large, dull, fibrous fruits of the "ruminant-rodent-elephant syndrome" (Gautier-Hion et al., 1985). In our videos, samango monkeys at both sites typically consumed at least 50% of fruits on *C. miniata* plants and both red and green fruit were eaten. Green fruits that had been maturing for eight months were likely almost ripe and contain seeds that will readily germinate (I. Kiepiel, unpublished data). Consumption of green fruits has been described for other neotropical primates (Barnett et al., 2012). That fruit and seed caches were not removed may have been a consequence of small sample sizes as secondary fruit, and seed

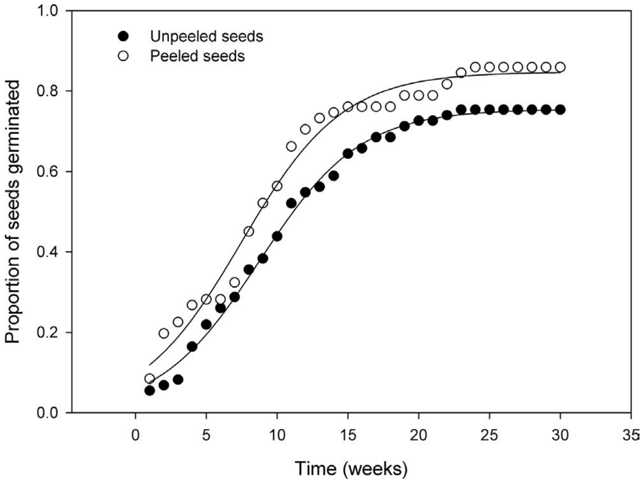

FIGURE 3 Changes in the cumulative proportions of germinated *Clivia miniata* seeds during 30 weeks for the unpeeled (solid symbols) and peeled (open symbols) treatments. The sigmoidal curves were derived using nonlinear regression

removal has been reported for other species dispersed by samango monkeys in South Africa (Seufert et al., 2010).

Monkeys de-pulped the fleshy fruit from the seed using their teeth and cleaned each seed in their mouth before spitting it out (Figure 1d, Video S1). It is unlikely that samango monkeys act as seed predators (Norconk, Grafton & Conklin-Brittain, 1998) as very immature fruit (prior to the stage when seeds can germinate) are not taken, and our observations of seed discarding are consistent with *C. miniata* seeds being toxic (Nelson et al., 2007; Viladomat et al., 1997; van Wyk et al., 2009). Previous studies have found that appreciable proportions of fruits consumed by *C. mitis* were unripe (Lawes, 1991; Linden et al., 2015).

Cercopithecus monkeys are known to disperse seeds by spitting which is a form of stomatochory (Corlett & Lucas, 1990; Lambert, 1999, 2001; Lucas & Corlett, 1998; Seufert et al., 2010). Seeds swallowed by primates are generally dispersed greater distances than those which are spat out (Chapman & Russo, 2007 and references therin). In central Africa, dispersal distances for seeds spat out by *C. mitis* were around 30-50 m from the parent plant (Rowell & Mitchell, 1991) and commonly around 10 m for those dispersed by red-tailed monkeys *Cercopithecus ascanius* (Lambert, 1999), which are longer distances than those observed in our study. Cercopithecines generally spit hard seeds > 4 mm in diameter (Corlett & Lucas, 1990; Kaplin & Moermond, 1998; Lambert, 1999). In South Africa, *C. mitis* swallows hard seeds up to 6 mm in diameter without destroying them (Linden et al., 2015). As the soft seeds of *C. miniata* are c. 13 mm in diameter, we infer that monkeys would chew them in order to swallow them if they were palatable. One study of *Strychnos mitis* S. Moore highlighted the efficacy of seed spitting in aiding germination, finding that 83% of seeds spat by *C. ascanius* monkeys successfully germinated, versus only 12% of unprocessed seeds (Lambert, 2001), which contrasts with our finding that seed germination in unprocessed *Clivia* fruits was almost as high as that for seeds removed from the fruit (Figure 3).

Our videos revealed that monkeys mostly process fruits close to the parent plant. Lacking visually bulging cheeks, the recorded fruit processing behavior for the most part appeared to be immediate consumption rather than fruit collection and cheek-pouching behavior. We did observe cases where monkeys placed a fruit or several fruits in their cheek pouches (Video S1) prior to moving off or ascending into the trees. This is a common behavior in monkeys and in one study in Afromontane forest in South Africa as many as nine of 25 species' fruits consumed by *C. mitis erythrarchus* were cheek pouched prior to processing (Linden et al., 2015). However, there is a possibility that cheek-pouching behavior is increased in the presence of human observers or predators. It may have been less frequent in our study because the observations were made with remote cameras and because predators are relatively rare at the study sites. Seeds of the majority of plant species are dispersed only a short distance from their parents (Wilson, 1993), and the *Clivia* seeds we could locate were, on average, dispersed only slightly further than 60 cm from the parent. It was however unlikely that we would have detected long-distance dispersal, given the cryptic nature of the seeds on the leaf strewn forest floor. We may also have underestimated dispersal distances in cases where the nearest plant to a seed was not the maternal parent. As cameras have a limited field of view they have limitations for determining seed dispersal distances and can bias results. Despite inherent drawbacks, camera trapping resolved the enigma shrouding the dispersal of the toxic and recalcitrant seeds of *C. miniata*. Some of the limitations of camera traps can be overcome by direct observations, but with the concomitant risk that presence of human observers alters monkey behavior. Ultimately, molecular markers could provide the most accurate measures of the actual dispersal distances for *Clivia* seeds.

Clivia miniata plants grow in microhabitats determined by soil and light conditions and seeds distributed too far away from these microsites may be at a disadvantage. Conversely seeds dispersed too close to the dense leaves of the parents would face direct competition from adults. Monkeys typically spit seeds just beyond the dark enclave created by the leaves of their parents, but still within the general microhabitat patch. Cheek-pouching would provide opportunities for occasional long-distance dispersal.

Much more work is required to understand the dispersal mechanisms of plants with unorthodox seeds. Some amaryllids, such as *Crinum* L. species, have unorthodox seeds packaged in fruits that remain green, and these probably rely on passive dispersal by water (Snijman & Linder, 1996). Dispersal by primates seems likely for other genera closely related to *Clivia* such as *Scadoxus* Raf. and *Haemanthus* L. which have large red fruits containing large unorthodox seeds. However, some *Haemanthus* species occur outside the natural habitat of samango monkeys, raising the possibility that other animals, such as baboons, vervet monkeys, rodents, or birds, also play a role in stomatochorous dispersal of unorthodox seeds.

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DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h6t7 g (Kiepiel & Johnson, 2019).

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REFERENCES

Barnett, A., Boyle, S. A., Pinto, L. P., Lourenço, W. C., Almeida, T., Sousa Silva, W. S., … Spironello, W. R. (2012). Primary seed dispersal by three Neotropical seed-predating primates (*Cacajao melanocephalus ouakary, Chiropotes chiropotes and Chiropotes albinasus*). *Journal of Tropical Ecology*, *28*, 543–555.

Chapman, C. A., & Russo, S. E. (2007). Primate seed dispersal: linking behavioral ecology with forest community. In S. Bearder, C. J. Campbell, A. Fuentes, K. C. MacKinnon, & M. Panger (Eds.), *Primates in perspective* (pp. 510–525). Oxford: Oxford University Press.

Corlett, R. T., & Lucas, P. W. (1990). Alternative seed-handling strategies in primates: seed-spitting by long-tailed macaques (*Macaca fascicularis*). *Oecologia*, *82*, 166–171.

Crouch, N. R., Mulholland, D. A., Pohl, T. L., & Ndlovu, E. (2003). The ethnobotany and chemistry of the genus *Clivia* (Amaryllidaceae). *South African Journal of Botany*, *69*, 144–147.

Evenari, M. (1949). Germination inhibitors. *Botanical Review*, *15*, 153–194.

Garber, P. A., & Lambert, J. E. (1998). Primates as seed dispersers: ecological processes and directions for future research. *American Journal of Primatology*, *45*, 3–8.

Gautier-Hion, A., Duplantier, J. M., Quris, R., Feer, F., Sourd, C., Decoux, J. P., … Thiollay, J. M. (1985). Fruit characteristics as a basis of fruit choice and seed dispersal in a tropical forest vertebrate community. *Oecologia*, *65*, 324–337.

Kaplin, B. A., & Moermond, T. C. (1998). Variation in seed handling by two species of forest monkeys in Rwanda. *American Journal of Primatology*, *45*, 83–101.

Kiepiel, I., & Johnson, S. D. (2014a). Breeding systems in *Clivia* (Amaryllidaceae): late-acting self-incompatibility and its functional consequences. *Botanical Journal of the Linnaean Society*, *175*, 155–168.

Kiepiel, I., & Johnson, S. D. (2014b). Shift from bird to butterfly pollination in Clivia (Amaryllidaceae). *American Journal of Botany*, *101*, 190–200.

Kiepiel, I., & Johnson, S. D. (2019). Data from: Spit it out: monkeys disperse the unorthodox and toxic seeds of Clivia miniata (Amaryllidaceae). Dryad Digital Repository. https://doi.org/10.5061/dryad.h6t7 g

Koopowitz, H. (2002). *Clivias*. Portland, OR: Timber Press.

Lambert, J. E. (1999). Seed handling in chimpanzees (*Pan troglodytes*) and redtail monkeys (*Cercopithecus ascanius*): implications for understanding hominid and cercopithecine fruit-processing strategies and seed dispersal. *American Journal of Physical Anthropology*, *109*, 365–386.

Lambert, J. E. (2001). Red-tailed guenons (*Cercophithecus ascanius*) and *Strychnos mitis:* evidence for plant benefits beyond seed dispersal. *International Journal of Primatology*, *22*, 189–201.

Lambert, J. E. (2010). Primate seed dispersers as umbrella species: a case study from Kibale National Park, Uganda, with implications for afrotropical forest conservation. *American Journal of Primatology*, *71*, 9–28.

Lambert, J. E., & Garber, P. A. (1998). Evolutionary and ecological implications of primate seed dispersal. *American Journal of Primatology*, *45*, 9–28.

Lawes, M. J. (1991). Diet of samango monkeys (*Cercopithecus mitis erythrarchus*) in the Cape Vidal Dune Forest, South Africa. *Journal of Zoology*, *244*, 149–173.

Lawes, M. J., Henzi, S. P., & Perrin, M. R. (1990). Diet and feeding-behavior of samango monkeys (*Cercopithecus mitis labiatus*) in Ngoye Forest, South Africa. *Folia Primatologica*, *54*, 57–69.

Levey, D. J. (1987). Facultative ripening in *Hamelia patens* (Rubiaceae): effects of fruit removal and rotting. *Oecologia*, *74*, 203–208.

Levi, T., & Peres, C. A. (2013). Dispersal vacuum in the seedling recruitment of a primate-dispersed Amazonian tree. *Biological Conservation*, *163*, 99–106.

Linden, B., Linden, J., Fischer, F., & Linsenmair, K. E. (2015). Seed dispersal by South Africa's only forest-dwelling guenon, the samango monkey (*Cercopithecus mitus*). *African Journal of Wildlife Research*, *45*, 88–99.

Lucas, P. W., & Corlett, R. T. (1998). Seed dispersal by long-tailed macaques. *American Journal of Primatology*, *45*, 29–44.

Meerow, A. W., & Snijman, D. A. (1998). Amaryllidaceae. In K. Kubitzki (Ed.), *Families and genera of vascular plants* (pp. 83–110). Berlin, Germany: Springer Verlag.

Midgley, J. J., White, J. D. M., Johnson, S. D., & Bronner, G. N. (2015). Faecal mimicry by seeds ensures dispersal by dung beetles. *Nature Plants*, *1*, 15141.

Moore, P. D. (2001). The guts of seeds dispersal. *Nature*, *414*, 406–407.

Nelson, L. S., Shih, R. D., & Balick, M. J. (2007). *Handbook of poisonous and injurious plants*. New York, NY: The New York Botanical Garden, Springer.

Norconk, M. A., Grafton, B. W., & Conklin-Brittain, N. L. (1998). Seed dispersal by neotropical seed predators. *American Journal of Primatology*, *45*, 103–126.

Nyiramana, A., Mendoza, I., Kaplin, B. A., & Forget, P. M. (2011). Evidence for seed dispersal by rodents in tropical montane forest in Africa. *Biotropica*, *43*, 654–657.

Pearson, T. R. H., Burslem, D. F. R. P., Mullins, C. E., & Dalling, J. W. (2003). Functional significance of photoblastic germination in neotropical pioneer trees: a seed's eye view. *Functional Ecology*, *17*, 394–402.

Rowell, T. E., & Mitchell, B. J. (1991). Comparison of seed dispersal by guenons in Kenya and capuchins in Panama. *Journal of Tropical Ecology*, *7*, 269–274.

SAS Institute Inc. (2016). *SAS/STAT® 14.2 User's Guide*. Cary, NC: SAS Institute Inc.

Schupp, E. W. (1993). Quantity, quality and the effectiveness of seed dispersal by animals. *Vegetatio*, *107*(108), 15–29.

Seufert, V., Linden, B., & Fischer, F. (2010). Revealing secondary seed removers: results from camera trapping. *African Journal of Ecology*, *48*, 914–922.

Snijman, D. A., & Linder, H. P. (1996). Phylogenetic relationships, seed characters, and dispersal system evolution in Amaryllideae (Amaryllidaceae). *Annals of the Missouri Botanical Garden*, *83*, 362–386.

Swanevelder, D., & Fisher, R. C. (2009). *Clivia: Nature and nurture*. Pretoria, South Africa: Briza Publications.

Viladomat, F., Bastida, J., Codina, C., Nair, J. J., & Campbell, W. E. (1997). Alkaloids of the South African Amaryllidaceae. *Recent Research Developments in Phytochemistry*, *1*, 131–171.

Wilson, M. F. (1993). Dispersal mode, seed shadows, and colonization patterns. *Vegetatio*, *107*(108), 261–280.

Witmer, M. C., & Cheke, A. S. (1991). The dodo and the tambalacoque tree: an obligate mutualism reconsidered. *Oikos*, *61*, 133–137.

van Wyk, B.-E., van Oudtshoorn, B., & Gericke, N. (2009). *Medicinal plants of South Africa*. Pretoria, South Africa: Briza Publications.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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APPENDIX 3

Kiepiel and Johnson —*Biotropica* 51: 619-625. 2019. – Data Supplement S1

SUPPLEMENTARY METHODS

STUDY SPECIES AND SITES.—We chose study sites based on a number of criteria; 1) large populations of *C. miniata* (some populations are sparse and plants and scattered over wide distances); 2) good levels of *C. miniata* fruiting (some populations exhibit poor fruit set due to dense forest and inadequate lighting); 3) the presence of resident troops of *C. mitis labiatus*; 4) Coverage of both Coastal-Lowland and inland Mistbelt forest. Forest sites were dominated by large mature tree species and understory vegetation is uncommon. *Clivia miniata* populations are found growing in leaf litter, on rocks or tree roots and form dense stands to the exclusion of other plant species.

Supplementary methods TABLE S1: Study site information.

Site	Coordinates	Forest type	Altitude (m asl)	Population size (plants)	Population area (m)	Number of plants monitored	Sampling effort (camera hours)
Umtamvuna Nature Reserve (UNR)	31°00' S, 30°09' E	Coastal-Lowland	150	150	80 x 70	15	625 days, 20 hours
Mbona Private Nature Reserve (MPNR)	29°17' S, 30°21' E	Mistbelt	1300	50	40 x 30	15	532 days, 15 hours
Bushwillow (BW)	29°18' S, 30°17' E	Mistbelt	1400	200	120 x 100	15	344 days, 12 hours

CAMERA TRAPS AND OBSERVATIONS OF FRUIT REMOVAL.—We focused cameras on *C. miniata* fruits in diverse microsites, including loose scree, rock, leaf litter; soil and among tree roots. We programmed cameras to respond to movement and recorded 30-s videos with an interval of 10 s between each video clip. We set cameras to additionally record a single photograph at the moment of triggering. We programmed camera traps for 24-h day/night sensing, with a high sensitivity for activation. We placed cameras as far apart as possible in each site and positioned them in different orientations (e.g. view from ground level; view from trees; view from rocks). We checked cameras at two week intervals when fruits were immature and few or no seeds were removed, and weekly once we observed fruit removal.

STATISTICAL ANALYSIS.—For nonlinear model fitting we fitted the sigmoidal function $P = \frac{A}{1+e^{-(w-x_0)/b}}$ to characterise temporal variation in the mean cumulative germination, where P is germination percentage (modelled as a binomial distribution), A is the upper asymptotic proportion, x_0 is the time (weeks) at which half of the seeds germinated and b determines the approach to the asymptote. We fitted four models, including the “full” model with separate estimates of all three parameters for the two treatments, and three “reduced” models with separate estimates for two parameters and a common estimate for the third. Likelihood-ratio tests were used to identify for which parameters of the full model, if any, significantly improve the explanation of variation in cumulative germination.

Supplementary methods TABLE S2: GLM and GEE model details.

Data	Response variable	Model	Probability distribution	Link function
Field (camera traps)	Number of fruits removed per plant	GLM	Poisson	Log
	Proportion of fruit removed per plant	GLM	Binomial	Logit
	Handling time per fruit (s)	GLM	Gamma	Log
	Estimated dispersal distance (cm)	GLM	Gamma	Log
	Proportion of seeds in mouth spat on camera	GLM	Binomial	Logit
	Proportion of fruits picked that were ripe	GLM	Binomial	Logit
Greenhouse	Proportion of seed germinating	GEE	Binomial	Logit
	Leaf length	GEE	Gaussian	Identity

APPENDIX 3

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Supplementary methods TABLE S3: Nonlinear model fitting details.

Model	-2loglikelihood	AIC	AICC	BIC	Δ_i	k	n
Separate A, b and x_0	277.8	289.8	291.4	302.3	1.7	6	60
Separate A and b	283.1	293.1	294.2	303.6	5	5	60
Separate A and x_0	278.1	288.1	289.2	298.6	0	5	60
Separate b and x_0	295.1	305.1	306.2	315.5	16.9	5	60

-2loglikelihood = twice the negative log-likelihood, AIC =Akaike's information criterion, AICC = corrected Akaike’s information criterion, BIC = Bayesian information criterion,

Δ_I = AICi – AICmin, k = number of estimated parameters, n = number of observations.

APPENDIX 3

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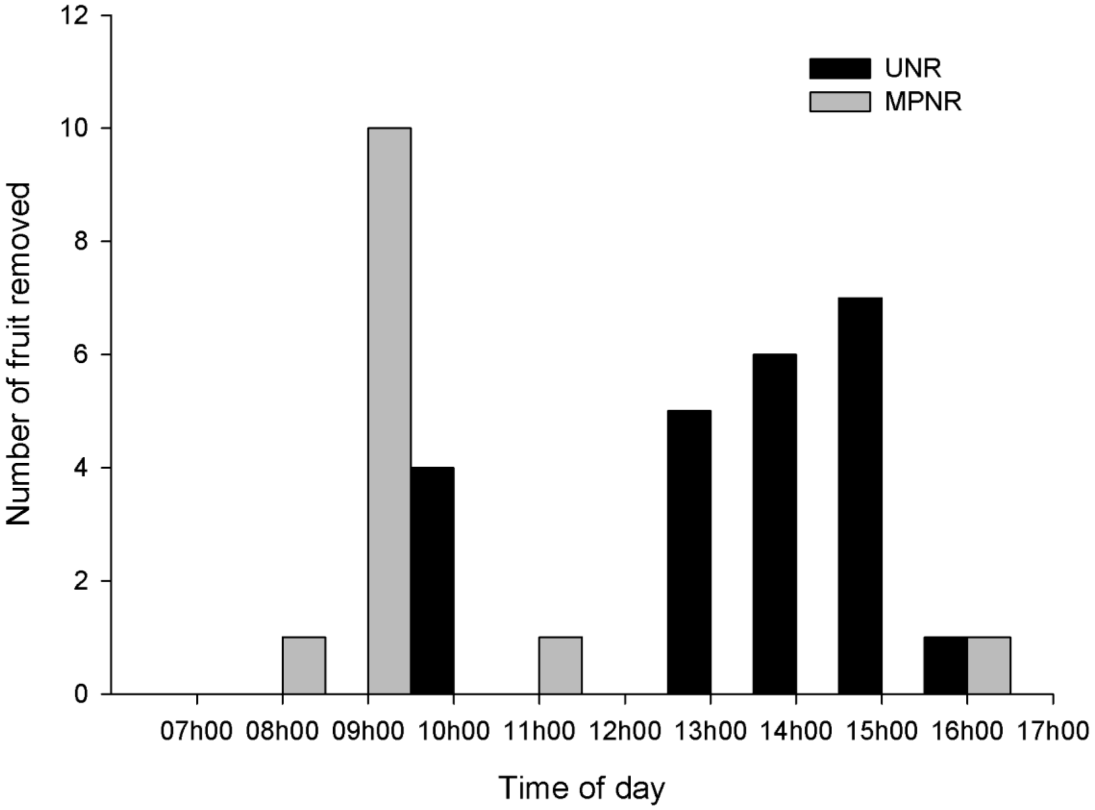
SUPPLEMENTARY FIGURES



Supplementary FIGURE S1: Camera trap observations of the number of *C. miniata* fruit removed by *C. mitis labiatus* in relation to the time of day, from two sites over the fruiting season of 2017.

SUPPLEMENTARY VIDEO

Supplementary video link:

https://www.youtube.com/channel/UCTD_vk0E7uzObqr63nP2Rgw

CHAPTER 6

DISCUSSION AND CONCLUSION

GENERAL DENOUEMENT

This thesis describes the breeding and pollination systems of two closely related and often sympatric *Clivia* species (Chapters 2 & 3). These results reveal divergent pollination systems in *Clivia* and provide evidence for a rare case of an evolutionary shift from bird to butterfly pollination (Chapter 2). This research adds to the growing body of literature in support of the commonly accepted idea that pollinator shifts are associated with key floral trait modifications and substantiates the role played by pollinators in angiosperm diversification (Chapter 2 & 4). Results from this thesis indicate that *C. gardenii* and *C. miniata* are principally self-sterile (due to LSI, severe early inbreeding depression or both), and that both species are reliant on pollinators for the production of seed (Chapter 3). This evidence taken together with the absence of pollen limitation (Chapter 3) and in the case of *C. miniata*, the high degree of pollinator efficacy together with foraging behaviour that likely limits geitonogamy (Chapter 2), is consistent with strong adaptive specialization between the two floral forms and their corresponding modes of pollination. These results highlight the potential for pollinator-mediated selection by identifying morphological and chemical traits that mediate butterfly interactions with *C. miniata* and propose a hierarchical process of evolutionary floral adaptations resulting in a specialized pollination syndrome associated with large swallowtail butterflies (Chapter 4). The seed dispersal ecology of *C. miniata* is elucidated, revealing adaptations for primate-mediated seed distribution, shedding new light on biotic dispersal systems of Amaryllidaceae with unorthodox seeds (Chapter 5).

This concluding chapter offers a précis of the research presented in earlier chapters of this thesis. Here, I expand on earlier discussions, highlight the limitations and the broader implications of this research, and discuss caveats and potential research streams.

POLLINATOR DRIVEN DIVERSIFICATION

Clivia gardenii and *C. miniata* exhibit distinct, highly specialized floral adaptations to different pollinator groups, providing compelling evidence for pollinator-driven diversification (Chapter 2, Grant, 1949; Grant and Grant, 1965; Stebbins, 1970). The clade presents an example of sister taxa with divergent floral forms, each pollinated virtually exclusively either by sunbirds and butterflies. Bird pollination in the earlier diverging species means that bird pollination is unambiguously optimized as the condition for the common ancestor of the two study species. This provides a model for evaluating the evolutionary transition from bird to butterfly pollination. Shifts from bird to lepidopteran pollination are not uncommon (Whittall and Hodges, 2007; Tripp and Manos, 2008; Gübitz et al., 2009), but to the best of my knowledge, this thesis reveals the first documentation of a shift from bird- to butterfly pollination (Chapter 2). The results of this thesis support the Grant-Stebbins model of pollinator-driven diversification, suggesting that pollinator shifts are associated with fundamental adaptive floral trait modifications (Chapter 2 & 4, Grant, 1949; Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006). Differential pollinator utilization as a result of pollinator heterogeneity (i.e. the “pollination climate”), provides a well-ordered postulation for speciation through adaptive specialization to the most abundant and effective pollen vector in *Clivia* (Grant and Grant, 1965; Stebbins, 1970). Here, pollinator spatiotemporal heterogeneity plausibly resulted in a pollination shift, producing significant adaptive specialization, selection for which favoured the floral traits underpinning adaptation to butterfly pollination - with the subsequent divergence in flower morphology and phenology presumably facilitating speciation through reproductive isolation (Grant, 1949; Grant and Grant, 1965; Stebbins, 1970). The drivers of *Clivia* speciation, or at the very least floral diversification, may convincingly be attributed to adaptive specialization towards dissimilar pollinators, however attributing definitive causative factors to pollinator spatiotemporal heterogeneity remains more challenging. Evidence suggests that during the last eight million years, cyclical glacial and interglacial eras have resulted in significant climate changes in tropical Africa, resulting in the expansion and contraction, and consequent alteration of forest distribution patterns, the vegetation and ecology of which, has been influenced through the dynamics associated with these fluctuations (see Hamilton and Taylor, 1992). It is likely that this palaeoclimatic oscillation resulted in variability in the "pollination climate", with floral trait selection directed by the most abundant and efficient pollinator (Grant and Grant, 1965; Stebbins, 1970).

An alternative or allied hypothesis may however, be put forward. Johnson and Bond (1994) suggest that in the red-flowered *Aeropetes*-guild, butterfly pollination evolved from nectar robbing of ancestral bird-pollinated flowers. This scenario shows a striking similarity between the evolutionary shift in *Clivia*, where floral colour appears to have significantly facilitated the evolutionary transition owing to the shared utility of this trait in both bird and butterfly pollination (Chapter 2). Given the flexibility of butterfly learning and propensity of the animals to rapidly adapt to new rewards (Swihart and Swihart, 1970; Lewis and Lipani, 1990; Goulson and Cory, 1993; Weiss, 1995; Kandori and Ohsaki, 1996; Weiss, 1997; Weiss and Papaj, 2003), a similar scenario in *Clivia* is not implausible. This scenario may have occurred through a mutation in orientation, where the long proboscis of large butterflies such as swallowtails were able to more readily probe the more accessible semi-pendulous flowers. Subsequent pollinator-mediated selection in this situation, would have presumably driven the widening of the corolla from a tubular-shape, providing for increasingly greater floral access by smaller butterfly species, resulting in the evolution of a conical-trumpet shape. Elongation of the flowers likely facilitated wing pollination during settling visits, with selection acting on radial expansion of the corolla mouth to maximise pollen placement on the wings.

The most prominent and influential floral trait modifications to emerge during the putative shift from bird to butterfly pollination in *Clivia* appears to be those of orientation and shape (Chapter 2 & 4). *Clivia miniata* flowers are presented upwards in an open fashion, providing a landing platform for butterflies which use the lower inside-lip of the perianth as an initial point of contact when alighting. Experimental manipulation of *C. miniata* flowers from an upright- to pendulous orientation was enough to completely prevent butterflies from alighting or probing flowers, and moreover, prevented any visible stigmatic or anther contact (Chapter 4). This suggests that variation in floral orientation played a pivotal adaptive role during the putative shift from bird to butterfly pollination in *Clivia* (Chapter 2 & 4). This research highlights the role of floral orientation as a fundamental mechanical isolating mechanism (Grant, 1949; Stebbins, 1950; Dobzhansky, 1951; Fulton and Hodges, 1999; Campbell, Jürgens, and Johnson, 2016). Flowers in a pendulous orientation, lacking 'perch-like' landing platforms are not easily accessed by butterflies, insomuch as by the same token, sunbirds require perches within probing distances of flowers for efficient feeding (Appendix 5, Anderson, Cole, and Barrett, 2005). Feeding trials with sunbirds that were presented with rewarding arrays of morphologically corresponding (floral curvature) and mismatched artificial model flowers, demonstrated that the birds generally fed first on those flowers generating the quickest handling times (i.e. those which correspond morphologically best to bill shape relative to suitable orientation and perch availability), feeding on mismatched models when more effortless rewards (i.e. those yielding lower handling times) were depleted (Appendix 5). In this study, model shape strongly influenced perching position, and birds preferred to perch below downwards facing curved models whilst feeding. Downwards facing curved models with a similar curvature and orientation to pendulous *Clivia* flowers generated the quickest handling times. When suitable top-perches were removed, models of unsuitable shape and orientation that require perches from above in order to feed efficiently, become more vexing to the birds, which favoured those models whose rewards were more quickly obtained. This study corroborates the idea that the floral preferences of perching nectarivorous birds can be explained by the role of flower curvature and orientation in relation to suitable perch position (Pauw, 2019; Sonne et al., 2019). Orientation has been demonstrated to act as a strong floral signal, imposing ethological isolation (Fulton and Hodges, 1999; Campbell, Jürgens, and Johnson, 2016), and reductions in visitation to flowers experimentally manipulated into a pendulous orientation have been reported (Fulton and Hodges, 1999; Giurfa, Dafni, and Neal, 1999; Ushimaru and Hyodo, 2005; Ushimaru, Kawase, and Imamura, 2006).

The diversification of pollination systems in *Clivia* likely represents an example of what Verne Grant expounded on from the classic works of Dobzhansky and Stebbins, and described as the '*Salvia* type' of mechanical isolation (Stebbins, 1950; Dobzhansky, 1951; Grant, 1994). This rather prevalent '*Salvia* type' of mechanical isolation functions in the presence of different floral forms, each of which possess adaptations to pollinators with differing morphology (Stebbins, 1950; Dobzhansky, 1951; Grant, 1994). The pendulous tubular flowers of *C. gardenii* and the upright trumpet-shaped flowers of *C. miniata* display strong adaptation to divergent pollination modes, the respective pollinators bodies of which, are clearly morphologically quite distinct. Pollination in *C. miniata* takes places chiefly via the wings of butterflies, whilst in *C. gardenii*, pollen is collected and deposited on sunbirds primarily via the head and base of bill (Chapter 2). In *Clivia*, floral isolation is further bolstered by what Grant describes as the '*Aquilegia* type' of ethological isolation, a by-product of mechanical isolation (Grant, 1994). Here, unsuited pollinators eschew mismatched floral forms owing to the handling difficulties encountered whilst foraging (Chapter 4; Appendix 5) – presumably only if alternative rewards are available. The '*Aquilegia* type' mode of ethological isolation is governed by the reward-to-cost ratio of foraging, where floral forms are avoided when low rewards are accrued, and preferred when foraging rewards are high (Grant, 1994). Floral diversification in *Clivia* therefore likely involved an initial divergence in floral morphology, with the resultant mechanical and ethological pollinator isolation bolstered by the advent of dissimilar flowing times.

The floral morphology of *C. miniata* imposes severe constraints on sunbirds by making it very difficult for a bird perched on the scape or pedicels to reach around into the corolla to probe for nectar without damaging the flowers. Although *C. miniata* flowers preclude birds from landing on the lip of the perianth as the tepals are unable to support the weight of birds, a malachite sunbird (*Nectarinia famosa*) was observed on a single occasion in 2009, robbing flowers of nectar in the Karkloof forests. Landing ungainly atop of the inflorescence, the bird was observed gripping the pedicels and robbing the flower of nectar by breaking through the corolla near the ovary. *Clivia gardenii* was observed to have been visited on a single occasion in 2016 in the KZN midlands by the emperor swallowtail *Papilio ophidicephalus*. The butterfly was observed landing on top of the inflorescence and walking tentatively down the corolla to reach the opening of the perianth. Probing was observed, but neither stigmatic nor anther contact was witnessed (flowers showed strongly exerted stigma and anthers). It remains uncertain whether the butterfly obtained nectar, but if this was the case, it was not an easy task and cost the animal a significantly greater handling time than that of *C. miniata*. It is likely that only bigger butterfly species would be able to do this, as smaller species would not have the required proboscis length to probe down to the nectaries. Anecdotal observations such as these are incredibly useful as they offer great insight in floral isolation. The dearth in observations of butterflies vising *C. gardenii* and birds that of *C. miniata* implies that the handling times involved in processing rewards from mismatched flowers are too great.

Clivia miniata occasionally flowers sporadically throughout the year, but natural hybridisation with sympatric pendulous species has only been reported for *C. caulescens*, the interspecific hybrid of which has been described as *C. x nimbicola* (Swanevelder, Truter, and van Wyk, 2006). Given the method sunbirds use to rob the flowers of *C. miniata,* they are an unlikely avenue for potential hybridisation. It cannot be ruled out that a sporadically flowering *C. miniata* was visited by a swallowtail butterfly, which subsequently visited *C. caulescens* (bee visitation also cannot be completely discluded). Far more so than butterflies, sunbirds are deft at robbing flowers that are morphologically unsuitable, being capable of easily piercing flowers with their bill – a skill unmanageable to butterflies. A noteworthy point of interest to add here is that *C. miniata* nectar is not bitter *per se*, but when the flowers are broken they exude a clear liquid, very bitter in taste, which like the rest of the plant itself, presumably contain some alkaloid component (Viladomat et al., 1997; van Wyk, van Oudtshoorn, and Gericke, 2009). A comparable effect occurs in *Clivia* fruits, which are quite palatable when ripe, but the seeds of which release a similar bitter liquid if damaged (Chapter 5). It may be that like the dark bitter-tasting nectar found in *Aloe vryheidensis* Groenew. which functions as a filter to exclude unsuitable pollinators such as bees and sunbirds from visiting the flowers (Johnson, Hargreaves, and Brown, 2006), this exudation functions in a similar fashion. Given the relative scarcity of floral resources available to sunbirds when *C. miniata* flowers, it is curious that birds do not rob the flowers more frequently. Mechanical constraints imposed on sunbirds by the upright-trumpet shaped floral form and a lack of suitable perch, together with unpalatable perianth exudations may strengthen the filtering of bird visitors, which are ineffectual pollinators.

Clivia miniata flower shape is such that the corolla mouth forms a funnel towards the centre of the flower into which butterflies can crawl whilst probing for nectar. Smaller butterfly species, constrained by the reach of their proboscis, must by necessity crawl further into the corolla than larger species. However, due to the conical nature of the flowers, smaller butterflies are limited by their wings in the extent to which they can progress into the corolla. This ostensibly acts as a filter, preventing the overutilization of nectar by smaller butterflies which may be relatively ineffectual at pollination but adroit as nectar robbers. *Clivia miniata* flowers are visited by and seemingly adapted to larger butterflies such as *Papilio* and to a lesser extent some of the bigger Pieridae (Chapter 2 & 4). South Africa has a number of other butterfly-pollinated amaryllid lineages, which show convergent evolution in their upright trumpet-shaped flowers (Johnson and Bond, 1994), and butterfly-pollinated examples of comparable floral morphology come from species such as *Lilium* L. in North America (Edwards and Jordan, 1992) and *Hemerocallis* L. in Eurasia (Hirota et al., 2012; Hirota et al., 2019). Butterflies pollinating *C. miniata* collected the majority of pollen on their ventral hind wings shortly after stigma contact (Chapter 2). The prominent ridges created by the butterflies' ventral wing venation form an ideal repository for pollen. As a butterfly moves deeper into the corolla tube of *C. miniata*, stigma contact may occur as the wings are folded to avoid damage. Following this, additional pollen may be deposited on the ventral side of the wings, the underside of the body, as well as any place on the body of the butterfly which is exposed to the dehiscing anthers. During butterfly brush visits, a large part of the wings are exposed to the stigma prior to floral alighting. This may explain why brush visits made by *P. dardanus cena* in the UNR were almost three times as effective compared to single settling flower visits. This study presents a contrast to previously reported swallowtail behaviour as swallowtails are thought not to settle during feeding visits (Henning et al., 1997; Woodhall, 2005). Feeding swallowtails have been reported to hover overhead a food source, rapidly beating their wings and extending their legs so that only their tarsus touch the flowers (Henning et al., 1997; Woodhall, 2005). *Clivia miniata* appears to have manipulated the behaviour of visiting swallowtails, bringing about a transition in their feeding behaviour from initial hovering to settling. The arrangement appears to suit both parties well and the butterflies are rewarded in energetic returns for their pollination services.

Based on data that are more anecdotal than those in this study, it has been proposed that other Amaryllidaceae in the South African fynbos, with red, brush-type flowers such as *Nerine sarniensis* Herb. and *Brunsvigia marginata* (Jacq.) W.T.Aiton are pollinated virtually exclusively by brush or 'inspection' visits made by the nymphalid *Aeropetes tulbaghia* (Johnson and Bond, 1994). The narrow funnel-shaped flowers and strongly exerted androecium of these species, so typical of brush-type flowers, ensures that during feeding visits, no anther contact whatsoever occurs (Johnson and Bond, 1994). Studies of the life history of *A. tulbaghia* indicate that 'inspection' visits may be associated with territorial behaviour which naturally increase in magnitude as the season progresses (Johnson and Bond, 1994). *Papilio ophidicephalus* males (along with most other species) are also very territorial, patrolling a meandering path of sunlit forest patches thought to be upwards of a kilometre in length (Henning et al., 1997). Another amaryllid, the blood lily, *Scadoxus multiflorus* Raf., has a large, globe-shaped umbel, containing numerous scarlet brush-type flowers, where the exposed anthers facilitate brush-pollination by hovering (or settling) swallowtail butterflies, which also collect pollen on the underside of their wings (Butler and Johnson, 2019 in press). The reproductive biology of *S. multiflorus* parallels that of *C. miniata* and moreover, highlights the diversity of floral forms attracting and sustaining swallowtail butterflies (see also Chapter 4).

Deposition of pollen onto *C. miniata* stigmas (including the number of pollen grains deposited in single visits) far exceeded the approximately 16 available ovules per flower (Chapter 3). Low fruit set and number of seeds per fruit even after supplemental hand-pollination in both *C. miniata* and *C. gardenii* suggest that pollen limitation is not prevalent but rather, fecundity appears to be resource limited (Chapter 3). Resource limitation in some populations of *C. miniata* may be so great that an individual may go a number of years without flowering (I. Kiepiel, unpublished data). Presumably plants in areas of denser shade suffer from greater resource limitation as they are more reluctant to flower than plants exposed to dappled sunlight (although see discussion on factors limiting fecundity). Other ecological aspects limiting fecundity such as severe grazing by antelope also need to be assessed in order to pinpoint the exact source of resource limitation (see Chapter 5).

The deposition of pollen on butterfly wings has been reported for several amaryllids (Johnson and Bond, 1994), lilies (Barrows, 1979; Corbera, Alvarez-Cros, and Stefanescu, 2018), mimusoid legumes (Cruden and Hermann-Parker, 1979), the flame Azalea *Rhododendron calendulaceum* (Michz.) Torr. (Epps, Allison, and Wolfe, 2015) and more recently in *Brunsvigia marginata, Cyrtanthus elatus* (Jacq.) Traub and *Scadoxus multiflorus* subsp. *katherinae* (Baker) Friis & Nordal (Butler and Johnson, 2019 in press). In this thesis, the positive correlation between butterfly wing scales deposited on stigmas and stigmatic pollen loads presents tangible proof for butterfly visitation (Chapter 2). Although analysis of lepidopteran scales on stigmas has been used previously as a proxy for visitation (Cruden and Hermann-Parker, 1979; Rodger, van Kleunen, and Johnson, 2013), to the best of my knowledge this was the first study to correlate the number of lepidopteran wing scales with stigmatic pollen loads and the first use of single-visit studies to test the hypothesis of pollen transfer via butterfly wings. Stigmatic scale loads can be used as a proxy for butterfly visitation but their predictive value fails when it comes to inferring the total number of butterflies which visit a single flower in the course of its life. It would be expected that frequent butterfly visitation to a butterfly-pollinated species should result in a strong positive correlation between the number of lepidopteran wing scales and the number of conspecific pollen grains found on the stigma. However, one cannot rule out the possibility of a single butterfly visitor depositing no pollen and many wing scales or *vice versa*. Identification of individual butterfly species based solely on histological scale analysis was attempted in this study but due to similarity (especially so for the swallowtails), could not be used as a reliable species indicator. Genetic barcoding of butterfly wing scales for species identification may prove to be a useful avenue in future studies. Extensive nocturnal observations of numerous populations of *C. miniata* ruled out the possibility of the wing-scales being of moth origin. Moths were not observed feeding on *C. miniata* despite their ubiquitous presence in forests after dusk.

Results from this thesis revealed that *C. gardenii* was found to be pollinated virtually exclusively by sunbirds (Chapter 2), corroborating several previous anecdotal reports of bird pollination in pendulous flowered *Clivia* (Koopowitz, 2002; Rourke, 2002; Manning, 2005). Field observations and selective exclusion experiments showed that sunbirds were highly effective pollinators of *C. gardenii*, with the orientation and morphology of the flowers precluding for the most part, all but birds and bees from visiting inflorescences. Lepidoptera such as hawkmoths and swallowtail butterflies which typically hover whilst feeding, cannot feed with any ease from the hanging pendulous flowers as proboscis extension in these animals occurs only along or perpendicularly below (not above) the animal's anteroposterior axis (but see earlier discussion). The absence of any large insect visitors to *C. gardenii* and evidence from selective exclusion experiments suggest that small insects contribute minimally to overall fecundity in *C. gardenii*. In order to feed effectively, birds preferably land on the scape of the inflorescence just below the hanging pendulous flowers. Where sunbirds have gripped an inflorescence, claw marks can clearly be seen on the peduncle. From this position below the flowers, the birds are free to probe for nectar in an upwards direction. This is consistent with numerous other studies showing that flowers with this type of floral syndrome are pollinated primarily by sunbirds (Geerts and Pauw, 2007; Botes, Johnson, and Cowling, 2008; Geerts and Pauw, 2009; Geerts and Pauw, 2012). *Clivia gardenii* floral traits wholly support purports of a bird-pollination syndrome, particularly one associated with the perching behaviour of sunbirds (Westerkamp, 1990; Anderson, Cole, and Barrett, 2005; Wester and Claßen-Bockhoff, 2006; De Waal, 2010; De Waal, Anderson, and Barrett, 2012; De Waal, Barrett, and Anderson, 2012). Plants frequently employ nectar rewards as floral adaptations to attract and sustain pollinators (Baker and Baker, 1975; Baker and Baker, 1983), and nectar is commonly of great importance in plant reproduction: often functioning as a primary floral reward (Simpson and Neff, 1983). Plant-pollinator interactions are commonly governed by availability and physiological attributes of nectar composition (Proctor, Yeo, and Lack, 1996). In both bird- and butterfly-pollinated *Clivia* species, nectar is the only floral reward available to pollinators. Sunbird-pollinated plant species generally produce a far greater amount of nectar than those species pollinated by butterflies (Johnson and Bond, 1994). This difference was also clear in *Clivia*, with *C. miniata* producing roughly four times less nectar than that of *C. gardenii* (Chapter 2). The present study also substantiates the fact that in contrast to nectar volume, sugar composition and nectar concentration tend not to vary substantially between bird and butterfly pollination systems in South Africa (Johnson and Bond, 1994; Johnson and Nicolson, 2008).

Flowers pollinated by animals with long mouthparts such hummingbirds or Lepidoptera, typically produce more dilute nectar with a higher ratio of sucrose to hexose, compared to those plants pollinated by animals such as bats, Diptera, perching birds and bees (which have comparatively short feeding apparatus), where the nectar composition is dominated by hexose (Baker and Baker, 1983; Freeman et al., 1984; Elisens and Freeman, 1988; Baker and Baker, 1990; Rusterholz and Erhardt, 1997). However, in some Lepidoptera such as the peacock butterfly *Inachis io* (Nymphalidae), females have been shown to utilise more balanced sugar solutions than males (i.e. 1 sucrose: 1 glucose: 1 fructose), and there is a strong preference to sucrose (5 sucrose: 1 hexose), and balanced solutions as opposed to hexose dominant (1 sucrose: 5 hexose) sugar combinations (Rusterholz and Erhardt, 1997). More general surveys indicate that the nectar concentration in butterfly-pollinated flowers tends to range from 15 to 25% (Baker and Baker, 1983; Cruden, Hermann, and Peterson, 1983), which is comparable to the nectar concentrations found in both hummingbird- and sunbird-pollinated flora (Johnson and Bond, 1994; Johnson and Nicolson, 2008). It has also been suggested that sugar concentrations between 20 and 25% ideally satisfy the energetic requirements for butterflies (Kingsolver and Daniel, 1979). Similarly, sunbirds such as *N. famosa* have been shown to prefer sucrose concentrations of 25%, favouring sucrose over hexose at such concentrations (Brown, Downs, and Johnson, 2010). In a rather extensive study conducted on 37 sunbird-pollinated *Erica's* in the South African fynbos, it was found that only eight species contained hexose-dominant nectar, whilst the majority of species comprised of sucrose-rich nectar (Barnes, Nicolson, and van Wyk, 1995). Furthermore, some sunbirds such as *Nectarinia chalybea* have been demonstrated to be able to practically entirely digest sucrose (Lotz and Nicholson, 1996), results of which contradict the previously held notion of passerine sugar preferences and the belief that passerines lack sucrase (Martinez Del Rio, 1990). The high proportion (almost two-thirds) of sucrose in *C. gardenii* nectar, suggests that this sugar is likely to be an important component of the visiting sunbirds diet (Chapter 2).

A shift from ancestral bird to more recent butterfly pollination in *Clivia* would have been one of relative ease with respect to nectar chemistry (Chapter 2). The similarity in nectar preferences between birds and butterflies suggest that the transition involved simply a reduction in the volume of the standing nectar crop. In terms of nectar concentration, this implies that bird-pollinated *Clivia* flowers are pre-adapted for a shift towards butterfly pollination. Despite the fact that both sunbirds (Brown, Downs, and Johnson, 2008) and butterflies (Rusterholz and Erhardt, 1997) have been shown to display some preferences with regards to sugar type, both groups will feed readily from hexose and sucrose solutions (Brown, Downs, and Johnson, 2008; Johnson and Nicolson, 2008). Inadequate nectar supplies, potentially unpalatable and toxic exudations from damaged flowers, awkward feeding positions and lengthy handling-times (see Appendix 5) would have presumably been enough to deter birds from regularly feeding on *C. miniata* flowers, where optimal foraging theory suggests they would seek elsewhere for more substantial rewards. The trade-off in terms of the reduction of large volumes of costly nectar would presumably come into being with the advent of bigger, more ostentatious, energetically expensive flowers. Pollinator-mediated selection would have presumably been directed towards adaptation of an upright and broader, more suitable landing platform. Ostensibly, putative pollen transfer involving the proboscis and heads of the butterfly was not as efficient as the wing pollination system or this floral architecture would have been retained and flowers may not have broadened or elongated to such an extent. Certainly, the butterfly wing presents a larger surface area for pollen transfer than does that of a head and proboscis combined.

Like nectar, floral colouration represents a trait which was little modified during the shift from bird to butterfly pollination in *Clivia* and one which may have offered a pre-adaptation to the evolutionary transition (Chapter 2 & 4). *Clivia miniata* and *C. gardenii* show very similar spectral reflectance patterns for parts of the flowers which are seen as orange to human vision (Chapter 2 & 4). It is unusual for flowers which are orange to humans to show significant UV reflectance and the UV component in *Clivia* blooms more than likely influences the colour perceived by pollinators, as both sunbirds and butterflies possess UV receptors (Eguchi et al., 1982; Endler and Mielke, 2005). It is well known that both butterflies (Knüttel and Fiedler, 2001) and birds (Cuthill et al., 2000) utilise ultraviolet light for the purposes of mate recognition, suggesting that the UV component of *Clivia* would be highly visible to both bird and butterfly alike. The primary modification in *C. miniata* floral colour was the evolution of a yellow throat situated at the base of the tepals (Chapter 2 & 4). This yellow target-like patch is very similar to that found in the butterfly-pollinated *Lilium philadelphicum* L. which likely serves as a nectar guide (Barrows, 1979), and is comparable in the swallowtail-pollinated *Hemerocallis fulva* L., which also has a UV component (Hirota et al., 2019). The yellow floral throat of *C. miniata* exhibits the highest UV reflectance produced by the flowers (Chapter 2 & 4). Results from choice arrays in this study indicate that some swallowtails such as *P. dardanus cena* are strongly attracted to orange model flowers with a yellow target bullseye pattern compared to plain orange models (Chapter 4). The use of artificial nectar guides or bullseye patterning also has been demonstrated to improve flower constancy and foraging efficiency in the cabbage white *Pieris rapae* (Kandori and Ohsaki, 1998).

Both sunbirds and butterflies rely strongly on visual cues for locating floral rewards, but it is likely that sunbirds rely exclusively on visual cues for locating flowers (Lewis and Lipani, 1990; Johnson and Bond, 1994; Ômura and Honda, 2005). This is reflected in the results of the present study which indicate that flowers of the two bird-pollinated *Clivia* examined are effectively unscented (Chapter 2). This substantiates evidence from numerous studies indicating that bird-pollinated flowers produce weak scent or lack scent emission entirely (Johnson, 1996a; Varassin, Trigo, and Sazima, 2001; Knudsen et al., 2004; Steenhuisen, Raguso, and Johnson, 2012). Although research suggests that some birds use scent in food location (Gomez et al., 1994; Nevitt, Veit, and Kareiva, 1995) and for navigation (Wiltschko, 1996), olfactory perception in the majority of birds is thought to be very poor (Bang and Cobb, 1968). Some butterflies are known to use scent together with floral colour as cues to locate reward bearing flowers (Andersson, 2003b). *Vanessa indica* (Nymphalidae) butterflies for example take their primary cues for floral visitation from colour, with scent playing an important role as a secondary cue (Ômura and Honda, 2005). Similarly, a combination of colour and scent has been found to stimulate visits by *P. rapae* to the flowers of *Brassica rapa* L. (Ômura, Honda, and Hayashi, 1999). However, in South Africa, some butterfly species have been shown to disregard scent and appear to rely mainly or solely on visual cues to locate flowers (Johnson and Bond, 1994). Results from this thesis indicate that scent plays an ancillary role to that of colour as an advertising signal (Chapter 4). My preliminary experiments with real flowers concealed by pollination bags (i.e. concealed floral colour, allowing for the emission of volatiles), indicated that floral scent alone was insufficient to attract butterflies (I. Kiepiel, unpublished data). Similarly, artificial scent supplementation to such concealed flowers elicited the same response (I. Kiepiel, unpublished data). Artificial flowers that where scent supplemented showed marginal increases in butterfly attraction, although an overall trend emerged in one season for scent preference (Chapter 4). Scent supplementation to real flowers on the other hand, resulted in a significantly higher number of approaches and brushes by *P. dardanus cena*. This highlights differential preferences of various butterfly species to scent and indicates that not only colour, but outline, or floral shape, may work synergistically as an advertising signal together with scent. The evolution of floral scent in the shift from bird to butterfly pollination in *Clivia* was likely of subsidiary importance to other floral traits, but one which was driven by the sensory preferences of some of the more prevalent pollinators of *C. miniata*.

Clivia miniata flowers produce a very simple blend of floral volatiles including benzaldehyde and benzyl alcohol, two compounds which are known to elicit electroantennogram (EAD) responses in some butterfly species (Topazzini, Mazza, and Pelosi, 1990). In a study investigating the occurrence of possible convergence in chemical composition of floral scent in 22 butterfly-pollinated plants, the authors came to the conclusion that there is no one specific compound that can be ascribed to the butterfly pollination syndrome (Andersson et al., 2002). This research did however indicate that benzenoids such as phenylacetaldehyde and 2-phenylethanol as well as monoterpenes of the linalool group were ubiquitous and therefore possibly distinctive of the syndrome (Andersson et al., 2002) - a propensity reciprocated in flowers pollinated by moths (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1995). The authors confirmed this using EAD techniques, where it was found that butterflies showed strong antennal responses to the aforementioned scent compounds (Andersson, 2003a). Results from this thesis showed a ubiquitous, albeit slight, EAD response to benzaldehyde from *P. dardanus cena* and *P. demodocus* (Chapter 4). It is likely that the sweet almond-like scent of benzaldehyde is attractive to a wide array of butterfly species and although not a fundamental advertising signal, plays a role in signalling in a number of species (Chapter 4). The simple blend of volatiles produced by *C. miniata* most likely indicate a lack of scent production in the common ancestor of the species and reflect the progressive shift in biosynthetic pathways which were underpinned by phylogenetic constraints in odour production. It may be speculated scent production was not a primary floral trait modification, but rather a latter adaptation, perhaps one that developed along with *C. miniata's* yellow bullseye pattern.

Along with floral trait modifications, it stands to reason that the shift from bird- to butterfly pollination in *Clivia* went hand in hand with a shift in flowering phenology. *Clivia gardenii* flower from March to June or autumn to winter (Austral), whilst *Clivia miniata* flower from August to November or late winter through spring. I speculate that the shift in flowering phenology was a trade-off between the synchronization of blooming with butterfly peak-flight periods and the coming of the spring rains. Rationale for this hypothesis is twofold. Firstly, *C. miniata* flowers do not tolerate rainfall as pollen is washed from the anthers, and the pollen is itself prone to rot and fungal attack during bouts of precipitation (I. Kiepiel, personal observation). Secondly, butterflies are not on the wing during overcast, cold or rainy weather which for all intents and purposes would render the flowers ineffectual in the absence of their pollinators. Although some swallowtails such as *P. nireus lyaeus, P. demodocus demodocus* and *P. dardanus cena* have year round flight periods, others such as *P. echerioides echerioides, P. euphranor* and *P. ophidicephalus* have double-brooded flight periods, all of which overlap with the flowering times of *C. miniata* (Woodhall, 2005; Swanevelder and Fisher, 2009). The butterflies larval foods are forest trees and the distribution of for example, *P. dardanus cena, P. echerioides echerioides and P.* ophidicephalus, show a startling similarity to the distribution of *C. miniata* (Woodhall, 2005; Conrad, 2008). The close proximity of butterfly brood-sites (and subsequent eclosure) to *C. miniata*, together with corresponding peak-flight periods suggests that butterflies would have been commonly found in the same habitat and in close proximity to the ancestral *C. miniata*.

A shift in flowering phenology would have catered for a diverse assemblage of butterflies, rather than the few species which are on the wing during the winter months. Although many Pierids such as *Nepheronia argia* and *Belenois zochalia zochalia* are on the wing year round, they do have peak-flight periods, neither of which coincide with the flowering of *C. miniata* (Woodhall, 2005). *Belenois zochalia zochalia* for example, is only found throughout the year in warmer areas (i.e. the coastal UNR site), and being one of the smaller visiting butterflies to *C. miniata*, was also rather ineffectual at providing stigmatic contact; doing so in only 27% of visits (Chapter 2). Some *C. miniata* populations may be found in Afromontane forests up to 1500 m (a.s.l.) and face considerably colder temperatures than coastal regions during the winter months, with temperatures dropping close to freezing during the night (I. Kiepiel, personal observation). During the winter, these elevated sites have far fewer butterflies than those of coastal forests. Spring flowering in *C. miniata* is closely synchronised with the general increase in butterfly activity and is suggestive of adaptation to a diverse butterfly pollinator assemblage (Chapter 2 & 4). Birds are commonly known to be generally more resilient to rain and cold than insects (Stiles, 1971). Shifts from bee to bird pollination have for example been speculated to enhance plant fitness when weather conditions, particularly temperature, are unsuitable for bee activity (Cruden, 1972). A shift from bird to butterfly pollination may have similarly accompanied a shift from colder to warmer temperatures, which resulted in an increase in butterfly abundance.

LATE-ACTING SELF-INCOMPATIBILITY AND THE DETERMINANTS OF SEED PRODUCTION

This thesis establishes that *C. gardenii* and *C. miniata* are capable of producing small quantities of seed through self-fertilization, but are principally self-sterile (Chapter 3). Horticultural reports of viable seed production through selfing (Duncan, 1999; van der Merwe, Robbertse, and de Kock, 2005; Swanevelder and Fisher, 2009), likely refer to either (or both) this restricted capacity or to selected plant lines chosen for their ability to self-fertilize, rather than being reflective of the mating systems of natural populations. Neither *C. miniata* nor *C. gardenii* were found in possession of conventional sporophytic or gametophytic self-incompatibility systems (GSI) acting in the style, which was evidenced by the similar growth of both self- and cross-pollen tubes into the ovaries of *C. miniata* and both the ovaries and the ovules in *C. gardenii*. Self- and cross-pollen tubes were regularly observed traversing the length of the pistil and entering the micropyle in both *C. miniata* and *C. gardenii*, but events in the ovule beyond this stage could not be seen due to poor image resolution. Pollen tube growth into the micropyle is typically one of the hallmarks of late acting self-incompatibility (LSI), but is also observed in self-sterility associated with inbreeding depression (Allen and Hiscock, 2008). Some species such as *Aconitum kusnezoffii* Rchb. (Ranunculaceae) have been found to use pre-zygotic LSI in combination with early-acting inbreeding depression, complicating categorization of these mating systems (Hao et al., 2012). Pre-zygotic LSI in *A. kusnezoffii* appears to be responsible in part for the rejection of self-pollen tubes, whilst early-acting inbreeding depression accounts for the termination of some of the seeds which could not be prevented through pre-zygotic LSI mechanisms (Hao et al., 2012). A more detailed investigation of egg nucleus and sperm nuclei fates, as well as zygote development would need to be undertaken in *Clivia* using confocal laser scanning microscopy to clarify the exact site and timing of self-rejection and to rule out the possibly of inbreeding depression as has been suggested for *Cyrtanthus* (Johnson, Butler, and Robertson, 2019).

In *Clivia*, the lack of differential pollen tube growth in both self- and cross-pollinated pistils when considered concurrently with low seed set results obtained from self-pollination treatments (Chapter 3), likely reflects the presence of either severe inbreeding depression or LSI (Seavey and Bawa, 1986). It has recently been suggested that the lower proportion of ovule penetration by selfed pollen tubes compared to that of cross-pollen tubes in *C. miniata* is indicative of some type of pre-zygotic stylar SI (Johnson, Butler, and Robertson, 2019). This suggests that some type of stylar signalling is involved in prezygotic rejection, which likely works in unison with a LSI system. LSI may include both prezygotic (Sage et al., 1999; Sage, Price, and Waser, 2006; Hao et al., 2012) and postzygotic mechanisms (Sage and Williams, 1991; Gibbs and Bianchi, 1993; Bittencourt, Gibbs, and Semir, 2003), and although inbreeding depression could not be ruled out in either *C. gardenii* or *C. miniata*, this incidence of a component of prezygotic stylar self-sterility in *C. miniata* is suggestive of an LSI system rather than inbreeding depression. Although *Habranthus gracilifolius* Herb. displays GSI (Streher et al., 2018), more closely related South African amaryllids such as *Cyrtanthus breviflorus* Harv. (Vaughton, Ramsey, and Johnson, 2010), *Cyrtanthus contractus* N.E.Br., *Cyrtanthus mackenii* Hook.f. and *Cyrtanthus ventricosus* Willd. show no evidence of conventional SI mechanisms such as GSI (Johnson, Butler, and Robertson, 2019). LSI is expected to show phylogenetic clustering, occurring in congeneric and confamilial taxa and the Amaryllidaceae is an example of a family which displays a large number of examples (see Gibbs and Bianchi, 1999; Vaughton, Ramsey, and Johnson, 2010; Gibbs, 2014 and references therin; Johnson, Butler, and Robertson, 2019). Some authors have suggested that LSI systems represent the ancestral condition of SI, being more prevalent in basal clades (Allen and Hiscock, 2008), but it has also been pointed out that a number of basal taxa show GSI (Gibbs, 2014). The African Haemantheae present a sound avenue for the investigation of SI systems and it is likely that more examples of LSI will emerge from other species of *Clivia* as well as *Haemanthus, Scadoxus, Gethyllis* and *Apodolirion*.

That both *C. gardenii* and *C. miniata* are effectively self-sterile, suggests that both taxa are dependent on pollination vectors for reproduction (Chapter 3). Autogamous pollination appeared to be infrequent in *C. gardenii* and *C. miniata* due to herkogamy and visitor behaviour (Chapter 2, although visitor behaviour for sunbirds was not published). Spatial separation of male and female reproductive organs in angiosperms has long been recognised as an evolutionary adaptation toward the prevention of inbreeding (Barrett, 2002) and the presence of herkogamy in both species is further indication of a mating system which is disinclined towards autogamous self-pollination. *Clivia gardenii* and *C. miniata* also possess dichogamy, specifically protogyny (I. Kiepiel, unpublished data). The life of a flower may be highly variable between different varieties, species, families, habitats, seasons, dates and breeding systems (Primack, 1980; Ashman and Schoen, 1994), but the female stage of both the life of an individual flower as well as overall flowering period in *C. gardenii* and *C. miniata* is the longest sexual phase (see Primack, 1985). It is reasonable to assume that since *Clivia* is fundamentally self-incompatible, protogyny serves as temporal barrier to the deleterious effects of self-pollination, and if LSI is present, ovule discounting. Together with reducing geitonogamous self-fertilization, protogyny has been demonstrated to enhance plant fitness (particularly male) by limiting pollen wastage incurred through pollen discounting (Harder, Barrett, and Cole, 2000).

The floral arrangement of *C. miniata* is such that the stigma is presented beyond the anthers, i.e. approach herkogamy (Kerner von Marilaun, 1902). This floral architecture promotes the deposition of viable outcross pollen first and foremost on the stigma, prior to the collection of pollen from the anthers by the pollinator (Chapter 2). However, approach herkogamy (Kerner von Marilaun, 1902) does not negate the deposition of self-pollen originating from geitonogamy and effective cross-pollination can only be assured if autogamy is prevented. Results from this thesis indicate that *C. gardenii* presents its stigmas before the anthers (i.e. stigma length is less than that of the anther length). The pendulous orientation of *C. gardenii* flowers suggests that if the anthers were presented closer to the perianth with the stigma below, the flowers would need to be pollinated within a few days during their female phase, prior to anther dehiscence in order to avoid sexual interference caused by autogamous self-pollination. Presumably, *C. gardenii* presents its anthers slightly below the pistil to prevent self-pollen from dropping onto the stigma. However, this is not always the case (see for example Chapter 1, Figure 1D) and stigmas may sometimes be found protruding far beyond the anthers.

Observations of stigmas *in situ* (i.e. microscopic evidence from open-pollinated stigmas taken from the field) indicate that a large initial deposition of pollen may actually form an impenetrable mechanical barrier to any further pollen deposition and prevent any consecutively deposited pollen from germinating (Chapter 3). It is likely that any mechanical obstruction such as dead pollen or debris encountered by viable pollen in this scenario would prevent desiccated *Clivia* pollen grains from hydrating on the stigma, which in turn would inhibit pollen germination (Nasrallah et al., 1994; Cheung, 1996). It has been suggested that *Clivia* pollen readily germinates within an hour of pollination and under favourable conditions pollen tubes reach ovules within 24 hours (van der Merwe, Robbertse, and de Kock, 2005). Results from this thesis indicate that at most, pollen tubes had only reached the mid-style after 24 hours in *C. miniata* (Chapter 3), but it is well established that a number of factors including temperature and atmospheric humidly can effect pollen germination and pollen tube growth (Cheung, 1996). *Clivia gardenii* and *C. miniata* pollen tubes only reached the ovaries after 48 hours (both self- and cross-pollen). The higher proportion of self- and cross-pollen tubes reaching the ovary of *C. gardenii* after 48 hours was most likely due to the reduced stigma length as compared to *C. miniata* (Chapter 2). It is likely that compared to *C. gardenii*, the greater style length in *C. miniata* (approximately 30 mm) correlates to a more time-consuming period of pollen tube growth through the pistil, which would be expected to involve greater resources. *Cyrtanthus contractus* which has shorter flowers than *C. miniata*, has been shown to exhibit similar pollen tube growth rates to that of *C. miniata* (Johnson, Butler, and Robertson, 2019). In this species, 24 hours was a sufficient length of time for approximately 50% of self- and cross-pollen tubes to reach the mid-style and at the 48 hour mark, pollen tubes had reached and began to penetrate the ovaries (Johnson, Butler, and Robertson, 2019). Approximately 20% of *C. contractus* ovules had been penetrated by pollen tubes after 48 hours, and the majority of ovules were penetrated after 72 hours, with no discernable difference between self- and cross-pollen (Johnson, Butler, and Robertson, 2019). In *C. breviflorus*, a species with far shorter flowers than its congeneric *C. contractus*, 30 hours was a sufficient length of time for the majority of self- and cross-pollen tubes to penetrate the ovules (Vaughton, Ramsey, and Johnson, 2010).

In species with LSI, seed production is expected to be compromised by ovule discounting following application of mixtures of self- together with cross-pollen (Waser and Price, 1991; Broyles and Wyatt, 1993; Sage et al., 1999; Sage, Price, and Waser, 2006; Vaughton, Ramsey, and Johnson, 2010; Johnson, Butler, and Robertson, 2019). Despite indication of a significant reduction in seeds per fruit following the application of mixtures of self- and cross-pollen, my results from pollen mixture experiments did not show clear-cut evidence for LSI in *C. miniata and C. gardenii*, (Chapter 3). This discrepancy arose from the results obtained from the application of mixtures of dead and cross-pollen to stigmas, which failed to produce more seeds than the flowers from treatments receiving mixtures of self- and cross-pollen, an intriguing result which has similarly been reported in *C. contractus* (Johnson, Butler, and Robertson, 2019). A mating system subject to LSI would be expected to show a reduction in seed set in treatments receiving any component of self-pollen through ovule discounting, which was not evident in this experiment (Chapter 3). Additionally, in pollen chase experiments involving the application of dead pollen prior to application of cross-pollen, seed set was limited compared to treatments where only cross-pollen was applied to stigmas. It remains uncertain if the reduction in seed set in flowers receiving mixtures of cross- and self-pollen or the depression in seed set in flowers following pollen chase treatments of self- prior to cross-pollen was due to ovule discounting or long distance signalling in the pistil. It may be that dead pollen on the stigma merely acts as a mechanical barrier to cross-pollen by preventing pollen germination. However, the interactions may be equally complex, perhaps involving some residual chemical signal from the self-pollen which was not destroyed by microwaving or possibly from a chemical signal produced via the microwaving process itself. Experimental design in this investigation (Chapter 3) could have been improved by the application of an additional treatment of dead cross-pollen in order to remove any conjecture surrounding the occurrence of microwave-stable sporophytic compounds that could reduce fertility.

Pollen mixture experiments in another South African amaryllid *C. breviflorus*, indicate that the application of dead and cross-pollen combinations yielded seed set comparable to that of live cross-pollen, although it should be noted that dead cross-pollen was used (Vaughton, Ramsey, and Johnson, 2010), whereas the present study used dead self-pollen (Chapter 3). Intriguingly, in pollen chase experiments with *C. contractus*, treatments involving the application of dead self-pollen prior to cross-pollination (48 hours subsequent), did not differ significantly from those in which live self-pollen was applied prior to cross-pollination (Johnson, Butler, and Robertson, 2019). This is comparable to the results presented in this thesis, where it would be expected that with a LSI system, prior application of self-pollen would result in a large reduction in seed set. Pollen chase experiments of this kind offer a sound approach in testing for LSI, and it would be expected that with this type of SI, 48 hours would be a sufficient length of time for significant (if not total) ovule discounting to occur. In *C. contractus*, two possible explanations for this uncharacteristic outcome have been suggested; either that some of the ‘dead’ self-pollen was not killed in the microwaving process (resulting in ovule discounting) or that excessive application of self-pollen resulted in stigma clogging, which acted as a mechanical barrier to subsequent cross-pollination (Johnson, Butler, and Robertson, 2019). Stigma clogging in this instance appears more plausible than experimental error, but it is intriguing that results from this thesis indicate similarly that the application of dead self-pollen prior to cross-pollen inhibited seed set compared to the application of pure cross-pollen. Given that controls for dead pollen were extensively used in both the *Cyrtanthus* research as well as this work in *Clivia* (i.e. zero seed set was found in applications of pure dead pollen), it appears more likely that either extensive stigma clogging or alternatively, that some type of stylar signalling is involved. The similar results obtained from these two closely related genera (i.e. *Clivia and Cyrtanthus*) allude to the possibility that stylar signalling or possibly even some form of GSI such as that found in *H. gracilifolius* (Streher et al., 2018) is involved in limiting fecundity.

As documented in this work in *Clivia* (Chapter 3), LSI is typified by low fruit set (i.e. less than 10%) and negligible seed production following self-pollination, yet it cannot be completely ruled out that *Clivia* species are self-compatible with very high levels of early acting inbreeding depression (Seavey and Bawa, 1986). However, other amaryllids such as *Narcissus triandrus* L. (Barrett et al., 1997), *C. breviflorus* (Vaughton, Ramsey, and Johnson, 2010) and *C. mackenii* (Johnson, Butler, and Robertson, 2019) also demonstrate leaky LSI, with self-fertilization leading to some degree of seed production. The majority of self-pollinated *C. gardenii* and *C. miniata* flowers abscised after approximately one week of hand pollination and this evidence supports the notion of LSI rather than early acting inbreeding depression (Seavey and Bawa, 1986). This period of time is also approximately the same time span in which senescing unpollinated flowers usually take to naturally abscise. However, it cannot be ruled out that the similar length of time taken for natural flower senescence and the senescence of hand self-pollinated flowers was due to the occurrence of duplicate recessive alleles which were incapable of driving ovule development. Self-rejection as a result of inbreeding depression does not typically result in uniform abortion of zygotes or seeds and this form of purging would be expected to manifest as a pattern of haphazard abortion (Seavey and Bawa, 1986). In species displaying inbreeding depression, total self-sterility may be found in certain individuals, but self-sterility in entire populations has yet to be documented (Waser and Price, 1991; Seavey and Carter, 1994). It has been suggested that full self-sterility resulting from inbreeding depression is unlikely as the genetic load necessary for total self-sterility is extraordinarily large (Waser and Price, 1991).

Unlike some species of Bignoniaceae and Bombacaceae in which seed set following selfing is practically non-existent (Gibbs and Bianchi, 1999; Bittencourt, Gibbs, and Semir, 2003), self-incompatibility systems in the Amaryllidaceae are known to be leaky (Barrett et al., 1997; Vaughton, Ramsey, and Johnson, 2010; Johnson, Butler, and Robertson, 2019). Up to 40% of self-pollinated *C. breviflorus* flowers have for example been shown produce fruit, although the number of seeds per fruit were very low (Vaughton, Ramsey, and Johnson, 2010). LSI has been demonstrated in *C. contractus* and *C. ventricosus*, but *C. mackenii* has demonstrated a striking similarity to *Clivia*, which is also characterised by partial self-sterility, and has been suggested by the authors to possess either a leaky LSI or alternatively strong inbreeding depression (Johnson, Butler, and Robertson, 2019). The partial self-sterility seen in *Narcissus* (Barrett, Lloyd, and Arroyo, 1996; Barrett, Cole, and Herrera, 2004), *Cyrtanthus* (Johnson, Butler, and Robertson, 2019) and *Clivia* (this thesis), support the view of a leaky LSI system (Barrett et al., 1997) or strong inbreeding depression, but given that LSI is expected to show phylogenetic clustering (Gibbs and Bianchi, 1999; Gibbs, 2014), points toward a LSI system rather than inbreeding depression. An important point to consider is that closely related taxa are highly unlikely to have individually accrued large genetic loads (Lipow and Wyatt, 2000), particularly so in cases of complete self-sterility (Waser and Price, 1991), and LSI is a trait which shows phylogenetic clustering and is preserved within families (Gibbs and Bianchi, 1999; Gibbs, 2014). To achieve unequivocal elucidation of the SI mechanisms responsible for governing fecundity in *Clivia*, genetic approaches need to be taken. *Clivia* take several years to flower from seed (Swanevelder and Fisher, 2009) and although testing for genetic SI could be done by raising arrays of sibling progeny to flowering stage in order to conduct diallel crosses as was done for *Asclepias exaltata* L. (Lipow and Wyatt, 2000), this would research would require a long term approach. Such crosses would be better suited to the confamilial *C. mackenii*, which has far shorter generation times than that of *Clivia* (Johnson, Butler, and Robertson, 2019).

Clivia gardenii and *C. miniata* populations typically show low levels of natural fecundity exemplified by low fruit and seed set relative to the available number of fruit and flowers per plant (Chapter 3). A plants reproductive output may be limited by a number of factors including, herbivory (Hendrix, 1988; Strauss and Zangrel, 2002), insufficient cross-pollination (Burd, 1994), resource limitation (Charlesworth, 1989) and in species with LSI, ovule discounting as a product of selfing (Waser and Price, 1991; Broyles and Wyatt, 1993; Sage et al., 1999; Sage, Price, and Waser, 2006; Vaughton, Ramsey, and Johnson, 2010). Results from this thesis indicate that low levels of natural fruit and seed set found in wild populations of *C. miniata* and *C. gardenii* were not significantly augmented by supplemental hand-pollination. This suggests that fecundity is not limited by the quality or quantity of pollen received by flowers, but is instead a product of maternal resource limitation (Burd, 1994; Harder and Aizen, 2010). Ovule discounting as a result of geitonogamy was ruled out as an influence on fecundity by evidence obtained from emasculation treatments, where the removal of anthers did not result in a significant increase in seed production. Additionally, observations of sunbirds visiting *C. gardenii* flowers and butterflies visiting those of *C. miniata*, indicated that these pollinators infrequently visit more than one flower per inflorescence, with the consequence that self-pollination as a result of geitonogamy is likely uncommon in any event (Chapter 2).

When cross-pollinated by hand, naturally occurring populations of *C. gardenii* and *C. miniata* failed to develop fruit in greater than approximately half of their flowers (Chapter 3). Similar pollination treatment in cultivated *C. gardenii* plants showed a striking contrast to that of wild populations, with almost all flowers of domesticated plants setting seed when hand cross-pollinated. This evidence again points to resource limitation as a factor responsible for reduced fecundity in wild populations of *Clivia*. The number of pollen grains deposited on open-pollinated *C. miniata* stigmas in the UNR was approximately five times greater than the average number of ovules available, however on average, seed development occurred in only of one out of every five ovules. The very low number of available ovules in *C. miniata* is a pervasive trait in the genus, and one which is likely allied with the reproductive costs involved in producing large unorthodox seeds (Chapter 5). In practice, this explains why a single application of pollen is more than adequate to fertilize all available ovules and for a plant to set full seed based on material resource capacity (Chapter 3). Additional applications of self-pollen should therefore have no effect, as not only will viable outcross pollen act as a mechanical barrier to germination of self-pollen (where larger amounts of initially deposited pollen would correlate to a greater mechanical barrier to any further pollen deposition), but the existing pollen tubes from viable cross-pollen will already have a temporal advantage in growth through the pistil. Butterflies and birds seldom visit more than a single flower on a plant (Chapter 2, although sunbird visitation data not published), and this behaviour minimises the chance for pollinator mediated geitonogamy. It therefore appears likely that low seed production in *Clivia* reflects resource limitation and resource trade-offs involved in the production of large fruit and large fleshy, unorthodox seeds, which in the case of *C. gardenii* can take up to 15 months to develop (van der Merwe, Robbertse, and de Kock, 2005). It has been suggested that low female fecundity has developed in long lived outbreeding species as a response to high energetic costs of maternal investment in the production of seed (Charlesworth, 1989). Considering plant and seed size, and the lengthy developmental period involved in reproduction, *Clivia* are certainly on the upper end of the spectrum when it comes to costly maternal resource investment.

I speculate that resource limitation in *Clivia* populations is a product of insufficient levels of light in forest habitats, and that this light-limitation results in maternal resource deficiency, preventing the full production of seed (Chapter 3). This line of thought is supported by suggestions from other authors that levels of fruit and seed production in natural populations of *C. miniata* and *C. gardenii* appear to be far lower than those produced by cultivated plants (van der Merwe, Robbertse, and de Kock, 2005). Although resource limitation arising from nutrient deficiencies cannot entirely be eliminated as a factor limiting fecundity, it seems more plausible that this is a product of suboptimal light diffusion through the forest canopy. Under ideal lighting, commercially grown *Clivia* will flower yearly, produce full fruit set, and may even produce more than one flowering scape per season (I Kiepiel, personal observation). This discrepancy in fruit and seed production between cultivated and wild stands of *Clivia* likely originates from the deficiency of light in forest habitats compared to the relatively high light levels found in greenhouse environments. In some of my ancillary greenhouse trials involving *Clivia* reared from seed, plants that were grown for several years in a nutrient poor coir substrate continued to display reasonable growth rates compared to those which were given supplemental nutrients in the form of fertilizers (I. Kiepiel, unpublished data). Several consecutive years of field observations indicate that *C. miniata* and *C. gardenii* plants which are positioned in dense shade do not flower each year. Conversely, plants situated in ideal conditions of dappled light rather than dense shade, flower yearly (I. Kiepiel, unpublished data). It is not uncommon to find entire populations in particularly dense shade, which year after year, exhibit little to no flowering whatsoever. The flowers of *C. miniata* in dense shade are also visited far less frequently by butterflies than those that are positioned in sunflecks under brighter lighting (I Kiepiel, unpublished data). Being primarily visually orientated animals, butterfly attraction to flowers decreases as flower visibility diminishes (see chapter 4). Butterflies in particular (more so than sunbirds), tend to avoid areas of particularly dense shade created by lianas and creepers (presumably because of their preference for warmer sunspots, and due to their delicate, easily damaged wings), finding the tangled forest undergrowth difficult to navigate. Given the exceptionally long-lived nature of *Clivia*, abstaining from flowering under these conditions would conserve valuable resources for future optimum reproductive opportunities (better light conditions provided by gaps in the canopy), which could otherwise be wasted fruitlessly. An alternative hypothesis would be that rather light-limited seed production, adequate light could itself be a flowering cue for the plants, and low-light conditions inhibit flowering in the first place.

Prior to an opening in the canopy (due to the falling of large mature trees), plants typically rarely exhibited flowering in deep shade, but in years subsequent to the development of tree-fall gaps, flowering appeared to be greatly stimulated by the additional sunlight penetrating the forest floor (I. Kiepiel, personal observation). Tree-fall gaps are known to be important factors for sustaining plant diversity in the forests of the tropics as they provide an opportunity for far greater amounts of light to penetrate the forest floor than would normally do so (Hubbell et al., 1999). The light from tree-fall gaps plays an important role in plant reproduction, and the growth of seedlings in both canopy and understory species (for review see Denslow, 1987). Sunflecks are commonplace in understory habitats and essential for understory plants (Chazdon, 1988) such as *Clivia*. Plant reproduction imposes heavy fitness costs on vegetative growth, particularly in understory species which typically encounter photon limitation (low light) or substantial photon fluxes (Clark and Clark, 1987). Decreases in vegetative growth can in turn limit or negate the reproductive efforts of a species and lead to a reduction in fecundity (Chazdon, 1988). The majority of understory forest species such as *Clivia*, exhibit vegetative (clonal) as well as sexual reproduction, and it has been speculated that clonal reproduction may offer a failsafe in light-limited environments because the establishment of seedlings in long-lived species is greatly constrained by the light limitations of the shady understory (Chazdon, 1988). Observations of plants flowering in full sun after the fall of large forest trees demonstrate that unrestricted sunshine severely damages the leaves of these individuals, but does not impede flowering. The flowers of these plants also suffer from sun damage and do not typically last as long as plants that are exposed to dappled light. Nevertheless, fruit set in these instances does not appear to be limited.

Because *Clivia* plants have the potential for clonal reproduction and are long-lived, SI presents an important mechanism for the prevention of inbreeding. However, the characteristic abortion of ovules at later developmental stages associated with LSI makes less intuitive sense for resource limited species such as *Clivia*. Compared to conventional SI mechanisms such as GSI in which ovule usurpation is not encountered, LSI presents a more costly energetic affair. The persistence of LSI despite the potential fitness costs of this mating system is reflective of selection for outcrossing despite these limitations, and more importantly, likely underpins the significant evolutionary restrictions involved in developing mechanisms of SI which are more efficient (Johnson, Butler, and Robertson, 2019).

Herbivory by *Tragelaphus sylvaticus* (southern bushbuck) appears to play a pronounced role in the ecology of some *C. miniata* populations (I. Kiepiel, unpublished data). Some populations which are not protected by rock scree and boulders may be so heavily grazed that all but the crown or the leaf bases remain. In extreme cases, the crown and apical meristem of the plants are so heavily grazed that they are completely destroyed, leaving little but the pseudostem intact. *Clivia miniata* populations growing in boulder fields formed by cliff falls are protected from antelope, which seemingly cannot traverse extremely rocky terrain. My field work revealed that entire populations of *C. miniata* were decimated by grazing in this way and that flowering was completely arrested for several years subsequent to this defoliation. Severe grazing was observed to frequently induce vegetative reproduction (I. Kiepiel, unpublished data), which highlights the low energetic cost of clonal, compared to sexual reproduction. Clonal reproduction under such severe predation becomes an important means of plant recovery as well as propagation. It typically takes several years for clonal offsets to naturally break away from parents, and this growth presents a resourceful mechanism for the damaged plants to maximise photosynthesis through spatial expansion to include multiple new growth points (apical meristems). In years of drought, herbivory becomes particularly acute in the dry winter months, and it is likely that given the toxicity of the plants (Crouch et al., 2003), *Clivia* is a resource which the antelope resort to as a famine food. Some *C. miniata* populations growing in unprotected (boulder free) environments are never predated, presumably because more palatable (less toxic) foods are available, or because antelope are not present, or population numbers are small (i.e. little competition). Curiously, *C. gardenii* populations do not appear to suffer damage from antelope and it may be that this species produces higher concentrations of toxic alkaloids than that of *C. miniata*. Similarly, the amaryllis borer *Brithys crini*, which can rapidly destroy domesticated *C. miniata* plants through flower and leaf feeding, also tends to avoid *C. gardenii*. Intriguingly, wild populations of *C. miniata*, unlike those of cultivated plants, do not appear to suffer much damage from the amaryllis borer (I. Kiepiel, personal observation). Although it could not be confirmed that the fruit consumption by *T. sylvaticus* captured on camera at the MPNR site included seed consumption (Chapter 5), given that the bushbuck consume vast amounts of leaf material that is presumably equally toxic as the seeds, seed predation cannot be ruled out. An investigation of seed and fruit toxicity through various developmental stages would assist in assessing the constraints on predation and the dynamics of seed dispersal in the genus (see Chapter 5).

FLORAL TRAITS AND ADVERTISING SIGNALS IN A SPECIALIZED POLLINATION MUTUALISM

Results from this thesis demonstrate that the floral traits of *C. miniata* function as important advertising signals for butterflies (Chapter 4). This thesis provides evidence for plant evolutionary adaptation to specialized butterfly pollination, offering support for pollinator-mediated floral trait selection (Chapter 2 & 4). These results demonstrate that the association between *C. miniata* and butterflies is that of a robust mutualism, where plant self-sterility requires the reward of nectar for pollination services (Chapter 2, 3 & 4). This relationship is an obligate mutualism for the plants, but the adaptable preferences and the ability of the butterflies to rapidly learn new floral rewards (Ilse, 1928; Swihart and Swihart, 1970; Goulson and Cory, 1993; Weiss, 1997; Kinoshita, Shimada, and Arikawa, 1999; Weiss and Papaj, 2003; Kandori et al., 2009; Kandori and Yamaki, 2012), means that the animals need not forage on *C. miniata* exclusively. The lack of visitation by other insects to wild populations and field-based arrays of model and real *C. miniata* flowers (I. Kiepiel, unpublished data), suggest specificity in these signalling cues, as observations reveal that this does not represent the absence of other potential forest pollinators. *Clivia miniata* flowers display many of the hallmarks associated with a classical butterfly pollination syndrome, including, vivid colour (including components of red), non-pendulous flowers, weak agreeable odour, diurnal anthesis, small quantities of dilute nectar, simple target-like nectar guides, herkogamy, and long floral-tubes with hidden nectaries which contain a high proportion of sucrose (Chapter 2, Faegri and van der Pijl, 1979). The flowers depart from the archetypal syndrome concept in that they do not possess the classical salverform corolla associated with a butterfly pollination syndrome (Faegri and van der Pijl, 1979). Although the flowers are tubular, they are not ubiquitously slender, and lack the abrupt, flaring (terminally spreading) tepals at corolla mouth, which is typified by the salverform morphology. Additionally, the rim of the blossoms are neither flat nor narrow (Faegri and van der Pijl, 1979). The flowers of *C. miniata* provide a distinct landing platform for butterflies in the lower tepals, above which the stigma protrudes. This may be bypassed when alighting takes place in the centre of a flower, but the strong exsertion of the style and radial anther arrangement allows for pollen transfer via the hind wings of the butterflies in the majority of visits (Chapter 2).

Butterfly-pollinated plants which similarly deviate from the classic syndrome, have previously been described in the South African flora and include *Cyrtanthus elatus*, *Cyrtanthus guthrieae* L. Bolus, *Brunsvigia marginata*, and *Schizostylis coccinea* Backh. & Harv. (Johnson and Bond, 1994). The red flowers of these species are examples of brush-type morphology, displaying strongly exserted stigmas and anthers, protruding from funnel-shaped corollas, which are similarly adapted to wing pollination by means of inspection visits of *A. tulbaghia* (Johnson and Bond, 1994). The floral shape of *C. elatus* and *C. guthrieae* are similar to *C. miniata*, except that flowers of the latter are much longer and have a less abruptly tapering corolla, with the result that the trumpet-shaped perianth forces butterflies to enter deep into the flower to feed (Chapter 2). Since probing can only occur when a butterfly moves deeper in the corolla, wing pollination occurs in both brush and settling visits. Wing pollination likely uses a significantly larger surface area of a butterfly's body compared to pollen placement on the proboscis, head or body. Presumably, the greater surface area lends itself to more room for error and may be an adaptation to a diverse array of butterfly species of varying sizes. The exceptional morphological variation of flowers in the *Aeropetes*-pollinated guild has been suggested to represent adaptation to diverse pollen-placement strategies which are necessary for a plant group sharing a single pollinator (Johnson and Bond, 1994). The morphology of *C. miniata* flowers represents the opposite scenario, where a uniform pollen-placement strategy has adapted to facilitate a diverse assortment of butterflies with varying wing sizes. Like a number of other South African amaryllids that show a high degree of self-sterility (Vaughton, Ramsey, and Johnson, 2010; Johnson, Butler, and Robertson, 2019), the mating system of *C. miniata* is associated with pollinator specialization which facilitates outcrossing (Chapter 3). It is possible that highly specialized pollination systems such as that found in *C. miniata* have adapted in response to pollen limitation and represent selection for outcrossing driven via pollinator-mediated floral trait selection (see Johnson, 1996b).

This thesis lends support to the predictive value of the pollination syndrome concept and illustrates a clear example of the utility of such predictions based on floral syndromes. However, even within the classical syndrome paradigm, plants often cater for more than one pollinator. Although phenotypic morphological expression may remain intact, populations often reflect discrepancies consistent with diverse pollinator requirements. Far be it from an irrefutable panacea for classifying pollinator-mediated selection, an important utility of the syndrome concept does involve its value in demonstrating convergent evolutionary adaptation and the targets of pollinator selection (Johnson and Wester, 2017). On a practical level, this ability to systematise pollinator functional groups in relation to floral traits streamlines experimental design and allows for more efficient hypothesis testing (Johnson and Wester, 2017). This thesis provides such an example, with the caveat that, the highly specialized nature of pollination systems in *Clivia* automatically bias the efficacy of the syndrome concepts predictive significance. It stands to reason that more generalized pollination systems would be expected to offer less fruitful grounds for extrapolative delimitation of pollination syndromes.

Results from this thesis reveal that colour functions as a primary advertising cue for the group of butterflies visiting *C. miniata* and therefore plays a central role in maintaining the mutualism between this assemblage (Chapter 4). This thesis adds to the large and growing body of literature highlighting the fundamental importance of floral colour as a signalling cue for foraging butterflies (Ilse, 1928; Swihart and Swihart, 1970; Lewis and Lipani, 1990; Goulson and Cory, 1993; Johnson and Bond, 1994; Weiss, 1995, 1997; Kinoshita, Shimada, and Arikawa, 1999; Ômura and Honda, 2005; Kocikova et al., 2012; Hirota et al., 2019). *Clivia miniata* floral traits conform to a 'diverse' butterfly pollination syndrome (Faegri and van der Pijl, 1979), in that the species is visited by several butterfly species from three families (Chapter 2 & 4). This butterfly assemblage showed marked spatiotemporal heterogeneity, fluctuating between coastal-scarp and inland mistbelt forests, as well as between seasons. *Clivia* floral colour presumably served as a major exaptation (Gould and Vrba, 1982), and remained little modified during the evolutionary transition from bird to butterfly pollination in the genus (Chapter 2). Pollinator-mediated selection for these hues was therefore likely driven by the visual preferences of this diverse butterfly group (Chapter 4). The geographical uniformity in *C. miniata* floral colouration when considered in conjunction with the spatiotemporal variation in butterfly diversity, indicates that pollinator heterogeneity exerts little regional selection pressure on colour variation. This may also explain the similar colouration found in the flowers of other African Haemantheae which are similarly pollinated by butterflies (Butler and Johnson in press).

The classical syndrome concept associates butterfly pollination with vivid colours including red (Faegri and van der Pijl, 1979). Both red (Swihart and Swihart, 1970; Scherer and Kolb, 1987a; Johnson and Bond, 1994; Kinoshita, Shimada, and Arikawa, 1999; Hirota et al., 2012) and orange (Kandori et al., 2009; Blackiston, Briscoe, and Weiss, 2011; Hirota et al., 2012; Kandori and Yamaki,

2012) are attractive to butterflies, yet innate preference for red is not as common as that of blue or yellow (see Kinoshita, Stewart, and Ômura, 2017 and references therin). Ômura and Honda (2005) point out that the extensive prevalence of blue as an innate chromatic preference may reflect the ubiquity of the colour as a cue for the majority of foraging butterflies. Although they are present in different insect orders, red receptors (Peitsch et al., 1992) are not abundant amongst the insects, and the perception of red appears to be far more common in the Lepidoptera (Bernard, 1979; Briscoe and Chittka, 2001). Red has been typically associated with bird-pollinated flowers (Grant, 1966; Raven, 1972; Faegri and van der Pijl, 1979), but the notion that groups such as hummingbirds show intrinsic predilections to red wavelengths is seen as misleading (Stiles, 1976; Goldsmith and Goldsmith, 1979). Even insects such as bees which neurologically lack red receptors are not, contrary to previous thought, completely red-blind, and do visit flowers in this wavelength (Chittka and Waser, 1997). It has been suggested that butterflies foraging on flowers of longer wavelengths such as red, benefit from the lack of bee visitation (Hirota et al., 2019), a reasonable hypothesis given that a large number of bee species avoid these colours (Rodriguez-Girones and Santamaria, 2004). Although bees were almost never seen visiting *Clivia* for pollen in the wild, observations of cultivated stands of *C. miniata* and *C. gardenii* in gardens around Pietermaritzburg showed that honey bees (*Apis mellifera*) were relatively frequent pollen collectors and may possibly contribute to pollination.

Given a variable "pollination climate" (Chapter 2 & 4), the utilization of an assortment of butterflies by *C. miniata* would presumably ameliorate any potential reductions in fitness stemming from discrepancies in the geographical or temporal pollinator mosaic (Grant and Grant, 1965). Adaptation to a diverse but highly specialized pollination assemblage would be expected to offer reproductive assurance, where pollination should be more readily guaranteed in the presence of numerous pollinating species. Results from this thesis demonstrated that pollen limitation was not a factor restricting fecundity in wild *C. miniata* populations (Chapter 3). *Clivia miniata* inhabits a number of different forest types and is the most widespread of all the *Clivia* species (Conrad, 2008). The area covered by *C. miniata* is roughly the same as that of all the pendulous flowered *Clivia* species combined, and it is likely that the putative shift from bird to butterfly pollination (Chapter 2), assisted the radiation of the species throughout the eastern regions of southern Africa. The mutualism between *C. miniata* and butterflies involves the recruitment of locally abundant pollinators, and results from this thesis show for example that *P. echerioides echerioides* and *P. ophidicephalus* were abundant pollinators in inland mistbelt forests, whilst in coastal-scarp forests, *P. dardanus cena* was the predominant pollinator (Chapter 2). *Papilio echerioides echerioides* is not found along the coast, and *P. ophidicephalus* has a very limited coastal range (Woodhall, 2005). In those species such as *P. dardanus cena*, which are not restricted by altitudinal gradients (i.e. not limited to either coastal or inland distributions), the observed differences in butterfly abundance between sites, may be explained by a number of factors including discrepancies in larval food availability or differing brood eclosure times.

Results from bioassays in this thesis revealed that simple conical *C. miniata*-shaped model flowers consisting of plain colours such as pink, blue, yellow, orange and red were highly effective visual signals to butterflies (Chapter 4). This corroborated preliminary findings of experimentation with flat paper disks (I. Kiepiel, unpublished data). Remarkably, by the same token butterflies were occasionally observed to be attracted to clothing and field-work apparel, particularly in the longer wavelengths of the visible spectrum (i.e. orange-red). Colour has been previously shown to be a critical signal in *V. indica* butterflies in foraging, taking preference over other adverting cues such as scent (Ômura and Honda, 2005). That butterflies use colour as a foraging cue is hardly surprising given the complexity and superlative range of their visual perception (Eguchi et al., 1982; Silberglied, 1984). Swallowtails are particularly visually adept, and the Japanese *P. xuthus* has true colour vision (Kinoshita, Shimada, and Arikawa, 1999) and a tetrachromatic visual system conveying some of the most exceptional wavelength discrimination of any animal (Koshitaka et al., 2008); a system not implausibly found in the southern African swallowtails. The colour preferences of butterflies are as diverse as their visual perception (for review see Kinoshita, Stewart, and Ômura, 2017), and together this complexity makes it difficult to attribute any one particular colour to a butterfly pollination syndrome (Faegri and van der Pijl, 1979). Confounding matters further, although butterflies show innate preferences towards certain floral colours (Ilse and Vaidya, 1956; Swihart, 1970; Scherer and Kolb, 1987b), they are also capable associative learning, quickly associating food rewards with colours (Swihart and Swihart, 1970; Lewis and Lipani, 1990; Goulson and Cory, 1993; Weiss, 1995; Kandori and Ohsaki, 1996; Weiss, 1997; Weiss and Papaj, 2003). While some species such as the southern African mountain pride *A. tulbaghia* are responsible for the pollination of a guild of entirely red flowers, preferring red model and real flowers alike (Johnson and Bond, 1994), such unambiguous colour preferences for a single colour are rare in butterflies.

Results from this thesis demonstrated that *P. dardanus cena* showed no preference in the number of approaches to either blue, yellow, orange or red models (Chapter 4). The swallowtails described in this thesis were at some point observed visiting flowers of varying colours in the forest, forest margin, as well as the surrounding grassland (I. Kiepiel, unpublished data). These included, pink flowered *Calodendrum capense* (L.f.) Thunb., *Dais cotinifolia* L. and *Watsonia mtamvunae* Goldblatt; blue flowered *Agapanthus praecox*, *Anchusa capensis* Thub., *Bolusanthus speciosus* (Bolus) Harms, *Hypoestes aristata*, *Plectranthus ecklonii* Benth. and *Plumbago auriculata*; yellow flowered *Bauhinia tomentosa* L., *Calpurnia aurea* (Aiton) Benth. and *Dietes bicolour* (Steud.) Sweet ex. Klatt and orange-red flowers of *Burchellia bubalina* (L.f.). Given that the pink flowers of *D. cotinifolia* and *C. capense* were visited by several butterfly species in the forest and flowering times of these two trees may overlap with *C. miniata* (November to December for *D. cotinifolia* and October to December for *C. capense*) it remains unclear as to why pink models were approached significantly less than blue, orange and red models (Chapter 4). It is possible that this was because the butterflies had yet to learn of floral rewards from the later flowering aforementioned tree species, and that pink does not represent an innate colour preference in *P. dardanus cena*. Observations of butterflies feeding on species with such varied floral colour, together with results from the five-colour array in this thesis, suggest that *P. dardanus cena* and other butterflies visiting *C. miniata* use a diverse spectrum of colours in foraging. This suggests that the butterflies are able to rapidly associate different colours with floral rewards as attraction to such a diverse array of colours is unlikely to represent merely innate colour preferences. The diverse morphologies of these flowers indicates that rather than floral shape, visual cues are taken more from vivid floral colouration. Indeed, it has been suggested that the evolution of the butterfly proboscis is an adaptation to rob nectar from a diverse morphological array of flowers (Wiklund, Ericson, and Lundberg, 1979).

Results from this thesis revealed that over two seasons, almost half of the butterflies that approached the real flowers of the *C. miniata* versus *C. miniata* var. *citrina* array alighted on them (Chapter 4). Alights to real flowers where far greater compared to those of models, which received only 6% in the five-colour model array, 36% in the colour pattern array, none in the target no target pair, 7% in the regular versus on-third size model array and 5% in the *C. gardenii* versus *C. miniata* model array. This suggests that whilst colour plays a vital role as an advertising cue in butterfly attraction, at very close distances such as those of inspection or brush visits, other cues are required to prompt alighting and stimulate probing behaviour. One possible explanation is that these cues are taken from contact chemoreception provided by the chemosensilla on the tarsi (Takeda, 1961; Fox, 1966). Further evidence of this may lie in the fact that when butterflies did alight on model flowers, visits were fleeting and probing was exceptionally rare (I. Kiepiel, unpublished data). The increased attraction to artificially scented model and real flowers indicates the importance of scent in strengthening signalling cues (Chapter 4). Results from this thesis showed that in all bioassays, the proportion of brush visits were far lower than the number of initial approaches, and lower still for the number of alights. However, it cannot be ruled out that visual details of flowers may also play a role in close range inspection visits and alighting behaviour. Another possibility is that the butterflies are able to perceive nectar or sugar rewards, which can only be done at close-range distances. Experimentation with field caught swallowtails showed that the butterflies will feed on rewarding (20% sucrose solution) blue and orange models identical to those used in the five-colour array (I. Kiepiel, unpublished data). However, it is difficult to disentangle the effects of starvation and desperation in such caged trials and this relationship needs proper assessment.

This thesis provides support for pollinator-mediated selection and helps to explain the persistence of orange floral colouration over that of the yellow flowered *C. miniata* var. *citrina* (Chapter 4). Whilst it is true that the anthocyanin based reddish hues of *C. miniata* have protective functions, offering defence from cold, water stress, and UV radiation (for review see Chalker-Scott, 1999), this thesis provides strong evidence for pollinator-mediated selection of floral colour. Being a forest species, and given that plants grow in dappled light, it seems unlikely that orange colouration reflects simply a protective adaptation to strong radiation. Although flowers in direct sunlight do become progressively redder in colour, there appears to be no marked loss of floral longevity in these situations (I. Kiepiel, personal observation). The persistence of numerous yellow flowered species growing in the full sun of the surrounding grassland supports this line of thought. Cold tolerance seems an equally unlikely factor for the dominance of orange over yellow, as coastal forests rarely drop below 15°C in the depth of the winter months (I. Kiepiel, personal observation). If cold protection was a factor influencing floral colouration, colour variation would be expected between low-lying coastal regions and inland forests at higher elevations. Protection from drought stress also seems unlikely as *C. miniata* flowering is synchronised with the spring rains, and the fleshy leaves, succulent pseudostem, and velamen-endowed roots allow the plants to hold appreciable amounts of water.

Results from the five colour-array in this thesis, revealed that orange models were strongly favoured over yellow models (Chapter 4). Similarly, in the colour-pattern array, orange was also strongly favoured over yellow. More importantly, real orange flowers of *C. miniata* were preferred over yellow flowered *C. miniata* var. *citrina* over two consecutive seasons. Results from these real flower arrays indicated that over two seasons, on average just under three-quarters of the butterflies approached the orange flowers of *C. miniata*. This evidence taken together with the immense scarcity of *C. miniata* var. *citrina* suggests that there is strong selection for orange colouration over that of yellow in wild populations. It remains unclear however, if this preference for orange reflects the innate preferences of butterflies or is learned from associating nectar with *C. miniata* floral colour. Given previous reports of the visual preferences of numerous butterflies for similar wavelengths (Swihart and Swihart, 1970; Faegri and van der Pijl, 1979; Scherer and Kolb, 1987a; Johnson and Bond, 1994; Kinoshita, Shimada, and Arikawa, 1999; Kandori et al., 2009; Blackiston, Briscoe, and Weiss, 2011; Hirota et al., 2012; Kandori and Yamaki, 2012; Hirota et al., 2019), it seems plausible that these colours are innate preferences. Although many butterflies can associate colour with rewards (Goulson and Cory, 1993; Weiss, 1995; Kandori and Ohsaki, 1996; Weiss, 1997; Kinoshita, Shimada, and Arikawa, 1999; Weiss and Papaj, 2003; Blackiston, Briscoe, and Weiss, 2011), this scenario seems less plausible, particularly in the swallowtails, which were frequently observed feeding shortly after eclosure. Evidence of butterfly emergence can be seen in a lack of wing-damage, and somewhat ungainly flight compared to older individuals (as flight muscles are conditioned). By the same token, older individuals show wing damage (particularly hind-wing), scale loss (and associated colour fading) and exhibit rapid, refined flight (I. Kiepiel, personal observation). Another line of thought it that of butterfly brood-site proximity to *C. miniata*. Laval foods for swallowtails consist of forest tree species (Woodhall, 2005), meaning that there would be a close proximity between butterfly eclosure and *C. miniata* flowers. The profusion of *C. miniata* flowers and the frantic drive for mate selection and oviposition sites suggest that butterflies need not venture far from *C. miniata* populations for their energetic requirements, which is suggestive of innate preferences rather than associative colour learning.

Red-orange floral colour with a small peak of UV reflectance is shared by all *Clivia* species, but *C. miniata* is unique in having a distinctive yellow throat (Chapter 4). This presumably reflects a mutation in the anthocyanin pathway, exposing the underlying yellow carotenoid pigments. Unlike the yellow colouration of *C. miniata* var. *citrina* which has not been selected for, the yellow bullseye target in *C. miniata* flowers persists across the species range (I. Kiepiel, personal observation). Results from the colour pattern array suggest that the presence of targets did not influence visitation of five butterfly species to these models (Chapter 4). However, results from the orange target no-target array showed that *P. dardanus cena* approached and brushed yellow throated models significantly more than those without a target (Chapter 4). These result are suggestive of species specific preferences for targeted nectar guides, however, the influence of context dependant preferences towards bullseye targets cannot be discounted (Kinoshita, Shimada, and Arikawa, 1999; Blackiston, Briscoe, and Weiss, 2011). In a similar study of *P. xuthus*, the authors suggest that bullseye patterns are used by the butterflies, although varying contrast intensity of bullseye patterning had no discernable effect on visitation (Hirota et al., 2019). In *P. rapae*, floral constancy and foraging efficiency are improved in the presence of bullseye targets (Kandori and Ohsaki, 1998) and in the papilionid *Troides minos*, the white coloured UV absorbing bract of *Mussaenda frondosa* L. forms an important long distance signalling cue (Borges, Gowda, and Zacharias, 2003). The persistence of these nectar guides in *C. miniata* implies selection, and preference for this patterning by *P. dardanus cena* supports the notion of pollinator-mediated selection for colour targets. Butterfly species such as *P. dardanus cena* may therefore be imposing selection against *C. miniata* var. *citrina* floral colours, firstly, because they are significantly less attracted to yellow, and secondly, because they prefer targeted bullseye patterns, which are lacking in the yellow form (Chapter 4).

Results from this thesis indicated that *P. ophidicephalus* preferred larger models over smaller ones (Chapter 4), which supports earlier work on the discriminatory preferences for larger model sizes of swallowtails such as *Papilio demoleus* (Vaidya, 1969). Here again it remains hard to completely eliminate the possible effect of reward conditioning, because smaller flowered species are visited by butterflies belonging to the *C. miniata* assemblage - including the emperor swallowtail (I. Kiepiel, personal observation). In a different array mimicking the morphology of butterfly and bird pollinated flowers, *P. ophidicephalus* similarly preferred *C. miniata*-shaped models over the smaller, pendulous *C. gardenii*-shaped models (Chapter 4). Taken together, results from these two arrays imply that the emperor swallowtail retains some degree of floral constancy and would likely choose *C. miniata* over similarly coloured species. The indifference of *N. argia*, *P. echerioides echerioides*, *P. euphranor* and *P. nireus lyaeus* to either *C. gardenii* or *C. miniata* models, could indicate a lack of floral constancy, but it is far more likely that these results indicate the strength of chromatic cues over other advertising signals. These results suggest that colour takes precedence over cues involving orientation and shape, and illustrate the importance of floral colour in the mutualism between *C. miniata* and butterflies. Results from the upright versus pendulous real flower array visited by *P. dardanus cena* and *P. nireus lyaeus*, suggest that in these species, colour cues similarly take priority over orientation. This bioassay yet again highlights species specific preferences and illustrates contrasts in the behaviour between *P. dardanus cena* and *P. nireus lyaeus*, and that of *P. ophidicephalus*.

Evidence from the upright versus pendulous real flower array, together with results showing that butterflies are similarly attracted to unsuitably orientated model flowers, illustrates a very important aspect of the shift from bird to butterfly in *Clivia*. Because colour is a fundamental primary advertising signal in the mutualism between butterflies, and *C. miniata* has been little modified in the evolutionary shift from ancestral bird pollination (Chapter 2), the only major floral trait modification required in the shift would have been that of the advent of upright orientation. An important result in the upright versus pendulous real flower array, was the effect of unsuitable orientation in floral isolation (Chapter 4). An increasing number of studies are showing that shifts in floral orientation can result in drastic changes in pollinator efficiency and have the potential to cause a shift in pollinators altogether (Fulton and Hodges, 1999; Campbell, Jürgens, and Johnson, 2016; Yon et al., 2017; Haverkamp et al., 2019). Manipulation of *C. miniata* flowers into a pendulous orientation resulted in the complete absence of alights to the flowers, an intriguing result given that butterflies theoretically could have landed on the lip of the tepals (Chapter 4). More importantly, it was not possible for the wings of the butterflies to physically contact the anthers or stigma of the flowers in this orientation as evidenced by the complete absence of anther or stigma contact in brush or settling visits. Although neither *P. dardanus cena* and *P. nireus lyaeus* showed a significant difference in choice during brush visits to either orientation, on the whole, both species more frequently brushed the pendulous flowers (i.e. exemplified in the overall choice significance of the statistical model). This appeared to be due to their immense curiosity, as the butterflies were visibly vexed in their inability to probe the flowers. This offers compelling evidence that the putative shift from bird to butterfly pollination in *Clivia* was underpinned by the transition from pendulous to upright floral orientation (Chapter 2). A modification in floral orientation alone, would have allowed large swallowtails to easily alight and probe the shorter ancestral *C. gardenii*-like flowers even without the widening of the corolla into a trumpet-shape. Flaring of the tepals at the mouth of the corolla would have allowed for smaller butterflies with proportionally shorter mouthparts to crawl deeper into the perianth to probe for nectar. Presumably, the energetic trade-off in producing larger flowers came with a fourfold reduction in the standing nectar crop (Chapter 2). It seems very likely that butterfly pollination in *Clivia* arose from a nectar robbing strategy in a similar manner as has been suggested for *A. tulbaghia* (Johnson and Bond, 1994). These results add to the comparatively sparse knowledge of the importance of orientation as a floral isolating mechanism (Fulton and Hodges, 1999; Campbell, Jürgens, and Johnson, 2016; Yon et al., 2017; Haverkamp et al., 2019).

This thesis provides evidence that scent production was associated with the shift from bird to butterfly pollination in *Clivia* (Chapter 2), and given that floral fragrance may alter pollinator behaviour and visitation rates (Faegri and van der Pijl, 1979), it is not surprising that scent could play a role in pollinator shifts and ultimately in speciation. Butterflies in the assemblage visiting *C. miniata* showed a high frequency of brush visits to flowers and models alike (Chapter 4). It is likely that scent signals assist in floral constancy and that the final stages of butterfly alighting and individual flower selection are determined by scent signals which direct the butterflies to newly opened flowers. It may also be that in addition to floral scent, butterfly visitation itself renders the depleted flower less attractive, perhaps due to a pheromone lingering on the flowers which the butterflies have learnt to associate with diminished nectar returns, as has been shown for bumblebees (Goulson, Hawson, and Stout, 1998; Stout, Goulson, and Allen, 1998) but see (Goulson, Chapman, and Hughes, 2001). Results from this thesis demonstrate that artificial scent supplementation slightly increased *P. nireus lyaeus* brush visitation to models, whereas other species showed no discernable significant preference (Chapter 4). In arrays consisting of real flowers, *P. dardanus cena* approached and brushed scent-supplemented flowers far more frequently than those of controls, suggesting that the combination of visual and olfactory cues creates a stronger advertising signal, than merely that of colour and shape alone. These results also illustrate species specific preferences in visual and olfactory cues. Butterflies show complex behavioural responses to the synergy of signalling cues. In some species, olfactory cues become redundant in the presence of innate colour preferences, yet when presented with colours that are not innately favoured, butterflies choose scented models (Ômura and Honda, 2005). *Heliconius melpomene* shows a remarkable relationship between learning and advertising signals, where olfactory foraging cues take priority in naïve butterflies, but are then progressively ignored through experiential foraging in favour of visual ones (Andersson and Dobson, 2003a). Although the results from this thesis using artificial scent supplementation offer only a crude insight into the role of scent, they do show that in at least two species, scent increased the attractiveness of flowers. As scent is of little importance to birds but important for butterfly attraction, the evolution of scent in *Clivia* could well have undermined the efficacy of bird pollination simply by increasing the frequency of butterfly pollination which resulted in selection for floral traits more suited to the newer more effective mode of pollination (Stebbins, 1970).

Electroantennographic evidence from this thesis showed that *P. dardanus cena* and *P. demodocus* exhibited clear responses to benzaldehyde (Chapter 4). Benzenoids are quite prevalent in butterfly pollinated species (Andersson et al., 2002; Dobson, 2006) and benzaldehyde has been found in more than half of the plant families studied (Knudsen et al., 2006). Benzaldehyde has been shown to elicit a very strong feeding response for a number of butterflies (Honda, Ômura, and Hayashi, 1998; Ômura, Honda, and Hayashi, 1999; Andersson and Dobson, 2003b; Ômura and Honda, 2005). In the cabbage white butterfly, *P. rapae*, similar results from EAD as well as proboscis extension reflex (PER) experiments, suggest that floral volatiles including benzaldehyde act as cognitive cues utilized in flower location and recognition at close-range distances rather than being long-range olfactory cues (Ômura, Honda, and Hayashi, 1999). Results from this thesis suggest that scent supplementation greatly increased the number of approaches by *P. dardanus cena* to real flowers, which indicates that scent could play a role at longer distances as well as closer ranges. However, the artificial scent blend that was used in these experiments was of a far greater strength to a human nose than that of the scent of *C.*

miniata flowers and these results may not be reflective of the close-range functions of scent given that odour emissions of this magnitude are unlikely. Antennal responses to benzyl alcohol and benzyl benzoate were recorded in male *P. demodocus* and female *P. dardanus cena* swallowtails (Chapter 4). Benzyl alcohol has previously been shown to be an important component of the scent of *Prunus yedoensis* Matsum., and has been found to induce PER in the papilionid *Luehdorfia japonica* (Ômura et al., 1999). Benzyl benzoate has been shown to elicit PER in *P. rapae* (Ômura, Honda, and Hayashi, 1999) and antennal responses (EAD) in the nymphalid *Inachis io* and the pierid *Gonepteryx rhamni* (Andersson, 2003a). Although it has been cautioned that EAG responses do not always match animal behaviour and feeding responses (Kinoshita, Stewart, and Ômura, 2017), results from bioassays indicate that butterflies do prefer scented model and real flowers in some situations (Chapter 4). Butterflies showed no aversion to scented model or real flowers at close-range, suggesting that the artificial *C. miniata* scent blend was not overwhelming. Benzaldehyde and benzyl alcohol are compounds which have been shown to be emitted not only by flowers, but also by vegetative parts (Andersson, 2003a). It may be that the unusually simple floral volatile blend of *C. miniata* (Chapter 2), reflects the comparatively recent adaptation and evolutionary shift from bird pollination. The lack of volatile complexity in *C. miniata* when compared to many butterfly pollinated flowers (Andersson et al., 2002; Andersson and Dobson, 2003a) is also suggestive of phylogenetic constraints in scent production, which is supported by the evidence of unscented bird pollinated congeners (Chapter 2). Given the potential phylogenetic constraints, the prevalence of benzaldehyde and benzyl alcohol in *C. miniata* scent may indicate the most efficient evolutionary pathway for scent production via adaptation of existing vegetative volatiles. It is likely that benzaldehyde and benzyl alcohol are quite common in other South African butterfly pollinated plants, particularly those in the Haemantheae such as *Scadoxus* and *Haemanthus* which likely suffer from similar phylogenetic constraints in scent production.

We have much still to learn of the visitation behaviour of lepidopteran pollinators (Weiss, 2001). Very few studies have assessed flower constancy in the Lepidoptera (Lewis, 1989; Goulson and Cory, 1993; Goulson, Stout, and Hawson, 1997; Pohl, Van Wyk, and Campbell, 2011) and very little evidence is available with regards to butterfly memory or how it is that floral constancy takes place in the first place (Lewis, 1986; Kandori and Ohsaki, 1996). The response of butterflies to positive floral rewards (i.e. honest signalling) has received considerable attention (Goulson and Cory, 1993; Weiss, 1995, 1997), however few studies have shown that butterflies can also respond to aversive stimuli in order to modify their foraging behaviour in the avoidance of low reward bearing flowers (Rodrigues, Goodner, and Weiss, 2010). Evidence suggests that learning new floral rewards is associated with significant costs (Snell-Rood, Davidowitz, and Papaj, 2011). The process of learning may accrue fitness compromises (Mery and Kawecki, 2004) and direct trade-off exists between learning and life history strategies such as overall fecundity (Snell-Rood, Davidowitz, and Papaj, 2011). The guild of butterflies vising *C. miniata* present a sound opportunity to further this knowledge.

DISPERSAL OF UNORTHODOX TOXIC SEEDS

The results of this thesis suggest that *Cercopithecus mitis labiatus* (samango monkey) is the primary disperser of *C. miniata* seeds in coastal-scarp and inland mistbelt forests of KwaZulu-Natal (Chapter 5). This thesis reveals the first report of a biotic seed dispersal system in the Haemantheae and highlights the need for further research on animal mediated dispersal of unorthodox seed. In general, the dispersal systems of unorthodox (recalcitrant) seeded plants are poorly researched, and much focus has been placed on seed physiology and desiccation sensitivity (Farnsworth, 2000; Berjak and Pammenter, 2008). This trend seems incongruous, given that recalcitrance is a trait which necessitates rapid seed germination (Berjak, Farrant, and Pammenter, 1989), and moreover, seeds generally require dispersal to negate density dependant constraints imposed by siblings and maternal parents (Janzen, 1970; Connell, 1971). Unorthodox seeds are usually big (characteristically larger than 4 cm), and over two thirds of recalcitrant seeded plant species produce only a single seed inside a fruit (Farnsworth, 2000). This makes unorthodox seeds unlikely candidates for widespread casual dispersal. Without the aid of dispersal vectors, large seeds would typically fall directly to the base of their maternal parents and are likely to suffer heavy competition. Seed removal and dispersal plays a vital role in the long term survival of species by shaping the dynamics and genetic structure of populations. Dispersal mechanisms assist plants in avoiding mortality resulting from density-dependant constraints (the “escape hypothesis”), in facilitating the occupation of open habitats when they become available for colonisation (the “colonisation hypothesis”), and in aiding seedlings to establish themselves in suitable local microsites (the "directed dispersal hypothesis"; Howe and Smallwood, 1982; Howe and Miriti, 2004).

Seed dispersal mechanisms in the Amaryllidaceae have for the most part been associated with water, and to a lesser extent wind, with little evidence for zoochory (Snijman and Linder, 1996). This is surprising, given that the diversification of the family is strongly allied with the evolutionary development of novel fruit and seed characters, which vary considerably (Snijman and Linder, 1996). Like many South African Haemantheae, the unmistakable, bright-red, fleshy berries of *Clivia* conform to the ‘bird-monkey seed dispersal syndrome’ (Gautier-Hion et al., 1985), and are strongly suggestive of animal dispersal (van der Pijl, 1982). Results of camera trapping from this thesis showed that samango monkeys typically consumed a minimum of half the available *C. miniata* fruits on a plant (Chapter 5). *Cercopithecus* monkeys are considered significant seed dispersers throughout the Afrotropics (Lawes, Henzi, and Perrin, 1990; Garber and Lambert, 1998; Seufert, Linden, and Fischer, 2010; Linden et al., 2015), and results from this thesis revealed that the large fruits of *C. miniata* were highly sought after by these primates, which readily ventured down from the canopy to obtain them. Although primates are well known seed dispersers of a great many species in tropical forests, the evolutionary and ecological importance of their activities is surprisingly poorly understood (Chapman and Russo, 2007). Despite the fact that frugivorous arboreal primates have been documented as important seed dispersal agents in tropical and subtropical forests, compared to the role they play in the canopy, little is known about their influence on understory plants, aside from their impact on secondary fruit and seed dispersal (Lambert, 1998; Lambert, 2002; Chapman and Russo, 2007; Seufert, Linden, and Fischer, 2010; Linden et al., 2015).

The diversity of forest dwelling primates in southern Africa is rather small compared to the rest of the Afrotropics (Chapman et al., 2002; Génin et al., 2016). Only two recognised subspecies of *C. mitis* occur in Southern Africa, namely, *C. mitis labiatus* I. Geoffroy 1843 and *C. mitis erythrarchus* Peters 1852 (Meester et al., 1986). *Cercopithecus mitis* are fairly widespread throughout the Afrotropics (incorporating several subspecies), and are found throughout central as well as eastern Africa, with distributions ranging latitudinally from Ethiopia into South Africa, and longitudinally from Angola to Somalia (Wolfheim, 1983). These primates occupy a range of habitats, including semideciduous and evergreen rainforest (Chords, 1986; Butynski, 1990), as well as tropical montane forests (Kaplin, 2001). Their diet varies considerably from region to region, with frugivory as low as 25.5% in Rwanda (Nyungwe Forest; Kaplin, 2001), and as high as 91% in South Africa (Ngoye Forest; Lawes, 1990b). Some authors have suggested that underpinning the large range distribution of *C. mitis* is the species' ability to utilize a high proportion of leaf material when food becomes scarce (Coleman and Hill, 2014). It has been demonstrated that fruit consumption in the diet of *C. mitis* significantly increases with temperature seasonality, whilst percentage animal matter holds the opposite trend (Coleman and Hill, 2014). This may offer a possible explanation for the large fruit component in the diets of South African guenons compared to those of the equatorial tropics. *Cercopithecus mitis* are suggested to be the most recently diverged (< 1 million years) of all the Cercopithecines (Dutrillaux, Muleris, and Couturier, 1988), and their distribution embodies the most southerly range of all the arboreal African guenons (Lawes, 1990a). *Cercopithecus mitis labiatus* has a distribution spanning the eastern parts of KwaZulu-Natal and the Eastern Cape Provinces of South Africa (Lawes, 1990a, 1992). *Cercopithecus mitis labiatus* is found in Afromontane type forest (subtype mistbelt forest; e.g. BW and MPNR sites in this thesis) as well as coastal scarp forest (e.g. Umtamvuna site in this thesis; note that Chapter 5 in its published form (Kiepiel and Johnson, 2019) erroneously refers to coastal-lowland forest and this should read coastal scarp forest throughout) of the Indian Ocean coastal belt forest (Lawes, 1990a, 1992). *Cercopithecus mitis erythrarchus* on the other hand, is found in Indian Ocean coastal belt forest, aside from that of coastal scarp forest, from northern KwaZulu-Natal, upwards into Mpumalanga and Limpopo in South Africa, as well as Mozambique and parts of Zimbabwe (Lawes, 1990a, 1992).

Results from this thesis revealed that *C. mitis labiatus* removed and consumed both green and red *C. miniata* fruit (Chapter 5). Green fruits are readily consumed by primates in the Neotropics (Barnett et al., 2012), and both adult and a juvenile *C. mitis labiatus* were observed consuming mature green *C. miniata* fruit. During early development, immature *C. miniata* fruit were eschewed by the monkeys. Any green fruit that was picked by monkeys, had been maturing for approximately eight months or more, and were likely almost ripe, containing seed that readily germinates (I. Kiepiel, unpublished data). This was evidenced from the fact that *C. miniata* seeds from somewhat immature green fruits (i.e. approximately six months), were able to germinate and establish in greenhouse conditions (I. Kiepiel, unpublished data). This is also consistent with the development of *C. mirabilis* seed, which takes between only four and six months to mature (Swanevelder and Fisher, 2009). This suggests that mature green fruits picked by monkeys are not at risk from being taken at a premature developmental stage, and seed derived from green fruits should readily germinate and establish given favourable conditions.

Results from this thesis demonstrate that *C. miniata* seed germination was positively correlated with fruit mass, and negatively correlated with the number of seeds per fruit (Chapter 5). Similarly, the length of a seedlings leaves (i.e. seedling growth), was positively correlated with fruit mass, and negatively correlated with the number of seeds per fruit. This evidence suggests that larger seeds have a selective advantage over smaller seeds. It remains unclarified whether seed from fully mature, green fruits are developed in size to the extent of red fruits. However, given evidence from greenhouse trials (I. Kiepiel, unpublished data), the exceptionally long period of seed development, the synchronised fruiting period, the fact that monkeys do not take immature fruit, and the relatively negligible time difference between when mature green and red fruits are consumed, it does not seem likely that the seeds of mature green fruit would experience any major resource differences (shortages), compared to those of red fruit. Indeed, aside from years of drought, and more than any other factor, seedling establishment appears to be most influenced by the dispersal of seeds to suitable forest microsites (I. Kiepiel, personal observation).

Footage from camera traps showed that samango monkeys mostly picked *C. miniata* fruit with their hands and very seldom plucked fruits directly with their mouth (Chapter 5). This behaviour may reflect that the dexterity of hand picking removes the risk of damaging the unpalatable seed by biting on the fruit too hard. Once picked, if a fruit was not immediately placed in a cheek pouch, the monkeys typically moved slightly away from the parent plant (pseudostem) to reposition themselves away from any constrictions imposed by the foliage, where they typically sat down on the forest floor and began processing fruits using the aid of their forelimbs. Seeds were rapidly and deftly de-pulped by the monkeys using their teeth, and often in quick succession. *Cercopithecus* are known to disperse seeds through ingestion as well as through seed dropping, where partial or total fruit de-pulping occurs (Kaplin and Moermond, 1998; Lambert, 1999; Linden et al., 2015). Seed spitting is an important mode of seed handling in *Cercopithecus*, which is also a characteristic trait of the Cercopithecinae subfamily; distinguishing them from all other non-human primates (Corlett and Lucas, 1990). Lambert (1999) has suggested that seed spitting in the Cercopithecinae is an adaptation which is facilitated by cheek pouches and the exceptionally well developed fruit processing capability of these primates. Further evidence of seed spitting in *C. miniata* was found in survey transects, where only undamaged seeds were observed on the forest floor (Chapter 5). The majority of these cleaned seeds were found within 1 m of prospective parent plants, corroborating video footage of monkey fruit processing behaviour in close proximity to parent plants. Seed located in forest surveys were very unlikely to have been a product of seed swallowing because the lack of testa surrounding *C. miniata* seeds would mean that if seeds were ingested they would have presumably been destroyed either by mastication or through the digestion process. It is likely that some seed was hidden by leaf litter and not accounted for in surveys. It also cannot be discounted that the nearest prospective parent plant was not in fact a seeds parent, with the result that seed dispersal distances may have been underestimated. Samango monkeys do not typically walk very far with fruit in hand, because they walk predominantly on all fours (I. Kiepiel, personal observation). If distances greater than a few meters need to be covered or if they are simply startled, the monkeys immediately make use of cheek pouches. Although cheek pouching behaviour was in the minority of fruit processing modes found in this research, another South African study of *C. mitis erythrarchus* found cheek pouching to be fairly common behaviour, with as many as nine of 25 species' fruits consumed by this subspecies found to be placed in cheek pouches prior to processing (Linden et al., 2015). However, the study used direct observation and not remote camera traps to assess fruit handling behaviour, and it cannot be ruled out that cheek pouching behaviour was magnified by the presence of human observers. Those seeds located at a distance greater than several meters from prospective parent plants were likely to have been cheek pouched and subsequently processed (Chapter 5). The cheek pouches of Cercopithecines are known to be able to hold almost the same capacity as the stomach of these monkeys (Fleagle, 1999), and this attribute has clear benefits when other troop members are feeding or the threat of predators is high.

Seed predation is not uncommon in neotropical primates (Norconk, Grafton, and Conklin-Brittain, 1998), but the unpalatable nature of *C. miniata* seeds are consistent with seed toxicity in the genus (Viladomat et al., 1997; Nelson, Shih, and Balick, 2007). Although *C. mitis* displays considerable regional dietary plasticity, and the ability to feed on foliage when food becomes scarce (Coleman and Hill, 2014), the alkaloid component of *C. miniata* (Viladomat et al., 1997; Nelson, Shih, and Balick, 2007), likely renders the seeds too toxic for the monkeys to consume. *Cercopithecines* in higher latitudes of the Afrotropics tend to spit out seeds greater than 4 mm but in South Africa, seeds of up to 6 mm are known to be swallowed (Corlett and Lucas, 1990; Kaplin and Moermond, 1998; Lambert, 1999). This puts the large (approximately 13 mm) seeds of *C. miniata* in the category of seeds that would be spat out if they possessed a hard seed coat. The exceptionally soft nature of *C. miniata* seeds implies that they would easily be ingested through chewing. This highlights the significance of seed toxicity as vital adaptation (or pre-adaptation), to primate dispersal of unorthodox seeds. It is likely that a great deal of other recalcitrant seeded Haemantheae such as *Scadoxus* and *Haemanthus* (which are likely also distributed by primates, and possibly rodents in the case of diminutive species of the latter) make similar use of seed toxicity in order to prevent seed ingestion. The tribe has is well known for the presence of isoquinoline alkaloids (Amaryllidaceae alkaloids) and toxicity is widespread throughout the Haemantheae (Wink and van Wyk, 2008). Lycorine, one of many toxic alkaloids in *C. miniata* (Crouch et al., 2003), has for example a known LD_{50} of 41 mg/kg (dogs), whilst *Haemanthus coccineus* L. contains the isoquinoline alkaloid montanine, which has a similar LD_{50} of 42 mg/kg (Wink and van Wyk, 2008). Both *Scadoxus* and *Haemanthus* are known to contain haemanthamine and haemanthidine

(Wink and van Wyk, 2008), which likely also play similar defensive roles in prevention of seed herbivory (as well as foliage).

Although monkeys occasionally cheek pouched fruit and moved off camera, the majority of *C. miniata* fruit was processed close to parent plants, with the consequence that most seeds were not dispersed very far (Chapter 5). Despite being in the minority of fruit handling behaviours, cheek pouching of *C. miniata* fruit may nevertheless facilitate a greater dispersal distance than terrestrial fruit processing (although see discussion of microsites below). However, long distance dispersal by monkeys seems unlikely. The seeds of the majority of plant species are dispersed only a short distance from their parents (Wilson, 1993). Based on camera evidence, *Clivia* seeds were dispersed only slightly further than 60 cm in both coastal and inland forests. However, camera traps have a limited field-of-view, and because of this, have limitations for assessing seed dispersal distances, and can result in bias. Nonetheless, camera trapping provides biologists with novel opportunities to study ecological interactions, and the use of camera traps to detect fruit consumption and seed dispersal is being used more frequently (Miura, Yasuda, and Ratnam, 1997; Nyiramana et al., 2011). In South Africa, camera trapping has previously been used for assessing secondary seed dispersal as a product of *C. albogularis* fruit feeding (Seufert, Linden, and Fischer, 2010). Although the limited field-of view provided by camera trapping comes with associated pitfalls of missing off-camera behaviour, it remains an incredibly valuable tool in documenting plant-animal interactions in remote locations. This is especially the case with regards to the study of cryptic or highly vigilant animals – the behaviours of which may be altered by, or missed entirely in the presence of an observer. Another benefit of automated cameras is that numerous cameras can be deployed over a wide distance, covering various microsites, microhabitats and multiple populations. A handful of camera traps set out for a few months can produce many hundreds of days' worth of data. Prior recording of metrics such as fruit number, plant height and interplant spacing, can provide meaningful results with regards to fruit removal, consumption and seed dispersal distances (Chapter 5). Although camera trapping is by no means fool proof, it will likely increasingly prove useful in ecological research.

Intriguingly, fruit dispersal events at the Bushwillow (BW) site were not captured despite the known presence of *C. mitis labiatus* populations (Chapter 5). Fruits that were recorded prior to camera placement were recorded as intact at the termination of camera trapping, and had become desiccated and turned brown in colour. It may be speculated that this was due to the vast size of *C. miniata* populations at this site compared to populations at the MPNR or UNR sites. The relatively short distance from the MPNR to BW site (approximately 8 km), implies that this lack of observation does not reflect regional differences or dietary preferences of these monkeys. Satellite imagery indicates that very narrow corridors of forest just barely link these two sites, and it is likely that agricultural development has resulted in extensive forest fragmentation of what was once one continuous forest. It seems likely that the consumption of only green fruit at MPNR was an indication of conspecific competition (Chapter 5). At MPNR, small populations of *C. miniata*, coupled with frequent sightings of at least two troops of *C. mitis labiatus* are suggestive of high competition pressure at this site. This may also explain why, on average, monkeys removed and consumed more fruits per plant at the MPNR site compared to the UNR site. Unlike the MPNR site, monkeys at UNR site, consumed fruit at varying stages of ripeness. *Clivia miniata* populations at the UNR site are much larger and more numerous than those of the MPNR site, and it is likely that the sweeter ripe red fruit (I. Kiepiel, personal observation) is actually preferred by the monkeys. Although *C. miniata* fruit development takes approximately one year (fruits ripen just before the flowering season begins in the Austral spring), frugivory was not observed until the later stages of fruit development (after approximately 8 months; Chapter 5). The abundance of mature green fruit at all three sites towards the end of winter, was an indication of negligible fruit consumption throughout early fruit development, the observations of which were corroborated by camera trapping.

Cercopithecus mitis is known to consume unripe fruit (Lawes, 1991; Linden et al., 2015), and it is likely that the toxicity of *C. miniata* fruit diminishes during later stages of fruit development, which may offer a possible explanation as to why immature green fruits are not consumed. The monkeys disregard for immature *C. miniata* fruit through early stages of fruit development, may allude not only to the toxicity of immature fruits, but may also represent the availability of more nutritious or possibly more palatable fruits in the forest. The fruiting phenology of *C. miniata* is such that ripening occurs in the dry season (roughly a month or two before spring rains commence), when arboreal fruits are not as readily available as in the summer months. The bitter element of the fruits, which are of an alkaloid nature (Crouch et al., 2003), certainly diminishes as the fruits begin to ripen and turn red (I. Kiepiel, personal observation), which is suggestive of a dispersal cue.

Fruit and seed caches placed on the ground appeared unappealing to monkeys and other frugivores alike, which for the most part, were observed in video footage walking over experimental caches with total disregard (Chapter 5). It cannot be ruled out that my scent was transferred onto the fruit and seeds whilst setting up these caches, but this seems unlikely to have been responsible for generating disinterest. That other frugivores such as Cape porcupines (*Hystrix africaeaustralis*) and chacma baboons (*Papio ursinus*) were observed similarly disregarding caches of both fruit and seed, remains somewhat of a mystery, but may be due to the low sample size of this cache experiment. Nonetheless, videos showed that both *H. africaeaustralis* and *P. ursinus* were disinterested in *C. miniata* fruit, and footage of both species passing plants in fruit (bearing mature green and red fruit) was frequently captured. Camera traps revealed that adult monkeys occasionally rejected fruits on a plant for reasons not apparent (I. Kiepiel, unpublished data). Fruit was held in the hand as if to assess the readiness of the fruit to break free from the pedicel, a behaviour that was not observed in juveniles. Intriguingly, this occurred with both green and a red fruit, making it unclear as to why these fruits were rejected.

Seeds spat out by primates are typically dispersed shorter distances than those that are swallowed (for review see Chapman and Russo, 2007). The influence of primate seed spitting on germination is generally poorly understood (Lambert, 2001; Balcomb and Chapman, 2003). However, one study of seed germination in *Strychnos mitis* S.Moore, demonstrated that seed spitting by *C. ascanius* was found to greatly aid germination (Lambert, 2001). This contrasts with results from this thesis, which indicated that germination of peeled seed was almost as high as that of unpeeled seed. This difference may be due to the fact that *S. mitis* seeds have a seed coat, whilst *Clivia* does not. Horticulturalists generally insist on peeling *Clivia* seed as a trusted method of commercial propagation, and unpeeled seeds are said to be more susceptible die-off from fungal contamination (Swanevelder and Fisher, 2009). It is possible that greenhouse germination trials may not reflect potential pathogens loads such as fungi and bacteria, which may be present under natural conditions in the forest understory. Germination trials shows that peeled *C. miniata* seed exhibited a quicker germination rate than that of unprocessed fruit, but there was no significant difference in growth between peeled and unpeeled seed treatments after 30 weeks.

This leads to the question of the evolutionary significance of investing in fruit which is energetically costly, when resource limitation is a factor limiting fecundity (Chapter 3). The answer to this question may be found in the fact that seeds have the best chance of establishment when they are spat out close to the parent plant in very specific microsites. The forest floor offers a particularly intricate microclimate, presenting a dynamic and potentially inimical environment to prospective seedlings. Seed that simply fall off the parent plant may incur competition with their parents for light and nutrients, whilst seeds which are distributed too far away from the patches and clumps of plants do not enjoy the benefits of ground stability, humus collection, and moisture retention which are offered by the roots of their parents and neighbours. Close examination of moisture levels in the forest (especially in the dry season), suggests that the soil moisture level in clumps of *Clivia* is substantially greater than those of the surrounding forest (I. Kiepiel, personal observation). Like orchids, *Clivia* roots have a velamen. It is this spongy velamen which gives *Clivia* its thick fleshy roots and one of the morphological traits that allows the genus to survive dry winter periods and times of drought. In the case of *C. mirabilis*, the velamen is of acute importance and is likely more defined in this species as an adaptation to the semi-arid Mediterranean climate in which it is found. The velamen in *Clivia* roots holds a great deal of water compared to the surrounding forest. *Clivia miniata* seed maturation and dispersal are timed to coincide with the spring rains, giving seedlings the best chance of establishment. However during the autumn and winter, there are often periods of many months when little or no rain falls in the forest. It is likely that during this time, the moisture collected by the roots of adult *Clivia* plants becomes particularly import to seedling survival. This hypothesis could easily be tested using *in situ* germination trials involving seed deposited at varying distances from conspecifics. *Cercopithecus mitis labiatus* appear to provide an elegant and seamless solution to this limitation by depositing seeds just beyond the dark enclave created by the leaves of their parents, but in close enough proximity for the seedlings to benefit from the moisture retained in the roots of adult plants. Most intriguingly, given the nature of the velamen, one cannot help ponder the possibility that, like orchids, the roots of *Clivia* share symbiotic relationships with bacteria or fungi, which assist in nutrient transfer.

In the far north of KwaZulu-Natal, where Afromontane forest transitions to that of Indian Ocean costal belt forest, it is likely that *C. miniata* seed dispersal is mediated by *C. mitis erythrarchus*. (Lawes, 1990a). Because the northernmost distribution of *C. mitis labiatus* ends in the far north of KwaZulu-Natal (Lawes, 1990a, 1992), it is likely that seed dispersal of *C. caulescens* is mediated by *C. mitis erythrarchus*. Like *C. miniata*, *C. gardenii* and the robust swamp gardenii *'C. robusta'* share overlapping distributions with *C. mitis labiatus* and their seeds are likely to be distributed by this subspecies. In more open habitats in which neither guenon is found, it is possible that other primates such as vervet monkeys *Chlorocebus pygerythrus*, are responsible for seed dispersal as they are similarly responsible for *Scadoxus* and *Haemanthus* dispersal (I. Kiepiel, personal observation). *Clivia mirabilis* habitats fall quite far from the distribution of either subspecies of *C. mitis* (Lawes, 1990a), and curiously, although the distribution of *C. pygerythrus* extends into both the Northern Cape and Western Cape Provinces (Haus et al., 2013), it does not quite overlap with *C. mirabilis* (Conrad, 2008), making it unclear as to whether the seeds of this *Clivia* are dispersed by primates. It also cannot be ruled out that birds such as the Knysna turaco (*Tauraco corythaix*), purple-crested turaco (*Gallirex porphyreolophus*) or trumpeter hornbill (*Bycanistes bucinator*) may be involved in seed dispersal of *Clivia*. Molecular markers could be employed to generate accurate measures of seed dispersal distances in *Clivia* and would greatly assist in determining the extent to which monkey's transport seed, and whether or not birds provide a means of long distance dispersal.

Some recalcitrant seeded amaryllids such as *Crinum* L. are adapted to water dispersal (Arroyo and Cutler, 1984; Howell and Prakash, 1990), which appears to be facilitated by the cork-covered outer layer of the fruits (Meerow and Snijman, 1998). Unlike *Crinum*, the ripe berries of *Clivia* fruit are fleshy and suggestive of animal dispersal. However, the robust form of *C. gardenii*, also known as the "swamp *gardenii*", is well adapted to waterlogged conditions and may be found growing in perennial swamps, with roots immersed in water. In very swampy conditions, the pseudostems may develop numerous buttress roots, which presumably help the plants to anchor and stabilize in the muddy substrate. These buttress roots also appear to stabilise the surrounding mud, allowing seeds to establish, preventing seedlings from being washed away by the increasing water levels which follow the spring and summer rains. Precocious seed germination in the "swamp *gardenii*" appears to be more common than that of its diminutive brethren, and it is not uncommon to find seedlings with leaves upwards of 5 cm in length still attached to the fruit (I. Kiepiel, personal observation). Swamp dwelling *C. gardenii* have very high levels of seedling recruitment (far greater than any other *Clivia* species), and seedlings are often found growing in clumps, presumably where fruit has fallen from the maternal parent (I. Kiepiel, personal observation). This also alludes to the fact that seedling establishment in the gracile form of *C. gardenii*, *C. miniata*, and presumably all *Clivia* species which are not found growing along water courses, is likely to be constrained heavily by water limitation. The peduncle height of some "swamp *gardenii*" often reach well over 1.5 m above the ground, and fruit simply dropping from these drooping pedicels has a good chance of escaping the umbrella-like canopy of shade, which is produced by the radiating leaf crown of the maternal parent. Significantly, seed of *C. gardenii* matures in mid-winter, giving seedlings enough time to put down roots in order to avoid being washed away as the rainy season approaches and water levels in the swamps rise.

Experimentation with *C. miniata and C. gardenii* fruit show that they are able to float when placed in water (I. Kiepiel, personal observation). However, the fruits become waterlogged fairly quickly and fruit pulp begins to rot, making this an unlikely dispersal strategy. Very few populations of *C. miniata* and *C. gardenii* are found exclusively growing along water courses (even the "swamp *gardenii*" grows in soils which are not swamp-like or waterlogged), however populations of both species may be found growing along water seepage, drainage lines, steep rocky outcrops and cliff faces eroded by water, streams, estuaries, rivers and waterfalls (I. Kiepiel, personal observation). *Clivia miniata* or *C. gardenii* plants do not grow with their roots immersed in water for the most part. However, heavy rainfall may result in above average seasonal fluctuations in water levels, leading to root emersion. The exception to this is of course the swamp form of *gardenii*, which thrives in perennially waterlogged swamps. Peeled seed of *C. miniata* and *C. gardenii* easily germinate in hydroponic trickle sand-culture systems and seedlings will readily establish (I. Kiepiel, unpublished data). *Clivia miniata* and *C. gardenii* also exhibit precocious seed germination and it is highly probable that some kind of linear, gravity assisted seed dispersal occurs along cliffs and steep watercourses, especially given that seeds do not necessarily require fruit de-pulping in order to germinate (Chapter 5). Unorthodox seed is a trait strongly associated with wet environments (Berjak and Pammenter, 2008). *Clivia* appears to conform to this suggestion, with the exception of the sun tolerant, Mediterranean adapted *C. mirabilis*, which is found in arid habitats (Rourke, 2002). Understanding the seed biology and dispersal of this species, presents yet another enigma in *Clivia* which begs resolution.

CONCLUSIONS AND RECOMMENDATIONS

Evolutionary shifts from bird to butterfly pollination are perhaps not uncommon and are simply underrepresented in the literature. Further examples are likely to emerge, particularly from the tropics where a rich diversity of bird and butterfly pollinators exist. Shared utility of floral traits indicates that some traits will function as pre-adaptations ('exaptation's', sensu Gould and Vrba, 1982) for such pollination shifts. The Haemantheae, with a large number of bird pollinated species and butterfly pollinated species (Butler and Johnson, 2019 in press) presents a number of possible contenders. The ability to transition from one system to another may even allow for the persistence of lineages that have experienced alterations to the local pollinator climate due to changing environments. It is likely that in the future, these more evolutionarily labile plant lineages will be better suited to face fluctuations in pollinator assemblages as a result of ecological impacts such as climate change or habitat loss.

A greater understanding of mating systems are required in *Clivia*. Late-acting self-incompatibly systems need to be explored further before any firm conclusion can be made with regards to self-sterility arising from inbreeding depression. Describing the mating systems of the southern African

Haemantheae presents an opportunity to determine whether LSI shows phylogenetic clustering at the genus, tribe or subfamily level. This research offers an important avenue to firmly establish the legitimacy of LSI. Since plant mating systems are inextricably linked to breeding systems, this research would also allow a greater integration between the two disciplines and greatly aid evolutionary understanding. Because commercial line breeding often requires extensive selfing, understanding and bypassing SI in the genus would greatly assist the development of new strains for horticultural purposes. Research into the genetics behind LSI is clearly an inevitable approach, which would expedite the understanding of this mating system.

The need is again highlighted to have a better resolved phylogeny of *Clivia*. The existence of *C. robusta* as discrete species is in itself highly debatable, and the proposal of a new Ngome *gardenii* species appears equally questionable (Spies and Spies, 2018), and haplotype data is far more suggestive of occasional hybridization. Although most available phylogenies place *C. miniata* as a product of the most recent bifurcation in the genus (Ran, Hammett, and Murray, 2001; Conrad, Reeves, and Rourke, 2003; Conrad et al., 2006; Conrad, 2008; Conrad and Snijman, 2011), the relationship between *C. robusta*, *C. gardenii* and *C. miniata* has not yet been fully resolved (Spies, Grobler, and Spies, 2011). Lumping *C. gardenii* (including *C. robusta*) and *C. miniata* into a single species complex (Spies and Spies, 2018) does not make sense in light of vastly dissimilar modes of pollination and floral isolation. A thorough taxonomic study involving material solely taken from wild populations is desperately needed. Combining DNA barcoding with microsatellites would be ideal for this purpose. This would also allow for the determination of the level of outcrossing in populations and additionally could provide the basis of an inquest into primate mediated seed dispersal distances. Given the commercial significance of the genus, whole genome sequencing would greatly assist this investigation and allow for an exhaustive platform for understanding the genetics behind flower colour, orientation, and scent production as well as elucidation of LSI and the mating system. The new microsatellite markers developed as part of this research have the potential to greatly assist in many of these studies (Appendix 4).

The visual system complexity of butterflies coupled with the adaptability and learning capacity makes the group an ideal model for examining plant-pollinator interactions in stable and fluctuating habitats alike. The relative ease of working with butterflies allows for progressively complex experimental methodology to be undertaken in a reasonably short period of time. *Clivia miniata* is a model species for examining specialized butterfly pollination and offers a strong platform on which hypotheses involving floral trait functionally may be further tested. The relatively simple floral shape, colour, colour patterning and scent allows for relatively uncomplicated disentanglement of signalling cues. The diversity of butterflies in the *C. miniata* assemblage present the opportunity to study species and genera specific visual and olfactory preferences. The methodology used in this thesis offers a fundamental starting point for exploring the functional significance of floral traits in this butterfly group, yet there remains much to be understood in species such *C. miniata* which show unidirectional obligate mutualisms to such highly adaptable generalists. Although definitive clarity on innate colour preferences will only be found through experimental determination, I hypothesise that blue and red are prevalent innate chromatic preferences in the southern African swallowtails. Further work needs to be conducted on naïve butterflies in order to determine cognitive aspects of innate preconditioned responses, learning ability as well as aversion and attraction to positive and negative reward bearing stimuli.

It is likely that rather than falling discretely into a 'bird-monkey syndrome' (Gautier-Hion et al., 1985), many African Amaryllids make use of a 'monkey-rodent' syndrome, where primary or secondary rodent dispersal is simply yet to be documented. Research is sorely needed in quantifying the dispersal services conferred by monkeys in African forests, particularly with regards to other members of the Amaryllidaceae. It is highly likely that the unorthodox seeds of the majority African Amaryllids are dispersed through non-ingestion by primates or rodents. This study presents a new avenue for research on seed dispersal interactions with primates, involving the reliance of terrestrial forest species on non-ingestion and dispersal within highly developed niches. As fundamental seed dispersal agents for topical forests, primates convey essential services to the ecosystems in which the inhabit, yet many primate species face widespread human threats, with more than 75% displaying population declines and roughly 60% endangered (Estrada et al., 2017). Much work is therefore urgently required to fully understand the dynamics of recalcitrant seed establishment and evolution of dispersal systems in the African Amaryllidaceae.

I conclude that shifts in pollination systems and the associated modification of suites of functional floral traits led to floral diversification in *Clivia*. Self-infertility in *Clivia* highlights pollinator dependence and pollination syndrome conformity reflects functional advertising signals. Gene flow appears to be governed by pollen flow and facilitated by pollinators rather than seed dispersal. Short distance seed dispersal by primates suggests restricted gene flow and genetic subdivision of populations, with mating and breeding system evolution a likely consequence of adaptation to isolated forest habitats.

REFERENCES

ALLEN, A. M., AND S. J. HISCOCK. 2008. Evolution and phylogeny of self-incompatibility systems in angiosperms. *In* V. E. Franklin-Tong [ed.], Self-incompatibility in flowering plants-evolution, diversity and mechanisms, 73-101. Springer, Berlin, Germany.

ANDERSON, B., W. W. COLE, AND S. C. H. BARRETT. 2005. Specialized bird perch aids cross-pollination. *Nature* 435: 41-42.

ANDERSSON, S. 2003a. Antennal responses to floral scents in the butterflies *Inachis io*, *Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae). *Chemoecology* 13: 13-20.

ANDERSSON, S. 2003b. Foraging responses in the butterflies *Inachis io, Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae) to floral scents. *Chemoecology* 13: 1-11.

ANDERSSON, S., AND H. E. M. DOBSON. 2003a. Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. *Journal of Chemical Ecology* 29: 2303-2318.

------. 2003b. Antennal responses to floral scents in the butterfly *Heliconius melpomene*. *Journal of Chemical Ecology* 29: 2319-2330.

ANDERSSON, S., L. A. NILSSON, I. GROTH, AND G. BERGSTROM. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Botanical Journal of the Linnean Society* 140: 129-153.

ARROYO, S. C., AND D. F. CUTLER. 1984. Evolutionary and taxonomic aspects of the internal morphology in Amaryllidaceae from South America and Southern Africa. *Kew Bulletin* 39: 467-498.

ASHMAN, T.-L., AND D. J. SCHOEN. 1994. How long should flowers live? *Nature* 371: 788-791.

BAKER, H. G., AND I. BAKER. 1975. Studies of nectar constitution and pollinator-plant coevolution. *In* L. E. Gilbert AND P. H. Raven [eds.], Coevolution of animals and plants, 100-140. University of Texas Press, Austin, USA.

BAKER, H. G., AND I. BAKER. 1983. Floral nectar sugar constituents in relation to pollinator type. *In* C. E. Jones AND R. J. Little [eds.], Handbook of experimental pollination biology, 117-141. Van Nostrand Reinhold, New York, USA.

------. 1990. The predictive value of nectar chemistry to the recognition of pollinator types. *Israel Journal of Botany* 39: 157-166.

BALCOMB, S. R., AND C. A. CHAPMAN. 2003. Bridging the gap: influence of seed deposition on seedling recruitment in a primate-tree interaction. *Ecological Monographs* 73: 625-642.

BANG, B. G., AND S. COBB. 1968. The size of the olfactory bulb in 108 species of birds. *Auk* 85: 55-61.

BARNES, K., S. W. NICOLSON, AND B. E. VAN WYK. 1995. Nectar sugar composition in *Erica*. *Biochemical Systematics and Ecology* 23: 419-423.

BARNETT, A., S. A. BOYLE, L. P. PINTO, W. C. LOURENÇO, T. ALMEIDA, W. S. SOUSA SILVA, B. RONCHI-TELES, et al. 2012. Primary seed dispersal by three Neotropical seed-predating primates (*Cacajao melanocephalus ouakary, Chiropotes chiropotes and Chiropotes albinasus*). *Journal of Tropical Ecology* 28: 543-555.

BARRETT, S. C. H. 2002. Sexual interference of the floral kind. *Heredity* 88: 154-159.

BARRETT, S. C. H., D. G. LLOYD, AND J. ARROYO. 1996. Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). *In* D. G. Lloyd AND S. C. H. Barrett [eds.], Floral biology: studies on floral evolution in animal pollinated plants, 339-376. Chapman and Hall, New York, USA.

BARRETT, S. C. H., W. W. COLE, AND C. M. HERRERA. 2004. Mating patterns and genetic diversity in the wild daffodil *Narcissus longispathus* (Amaryllidaceae). *Heredity* 92: 459-465.

BARRETT, S. C. H., W. W. COLE, J. ARROYO, M. B. CRUZAN, AND D. G. LLOYD. 1997. Sexual polymorphisms in *Narcissus triandrus* (Amaryllidaceae): Is this species tristylous? *Heredity* 78: 135-145.

BARROWS, E. M. 1979. Flower biology and arthropod associates of *Lilium philadelphicum*. *Michigan Botanist* 18: 109-115.

BERJAK, P., AND N. W. PAMMENTER. 2008. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. *Annals of Botany* 101: 213-228.

BERJAK, P., J. M. FARRANT, AND N. W. PAMMENTER. 1989. The basis of recalcitrant seed behaviour. *In* R. B. Taylorson [ed.], Recent advances in the development and germination of seeds, vol. NATO ASI Series (Series A: Life Sciences), vol 187. Springer Boston, MA, USA.

BERNARD, G. D. 1979. Red absorbing visual pigment of butterflies. *Science* 203: 1125-1127.

BITTENCOURT, N. S., P. E. GIBBS, AND J. SEMIR. 2003. Histological study of post-pollination events in *Spathodea campanulata* Beauv. (Bignoniaceae), a species with late-acting self-incompatibility. *Annals of Botany* 91: 827-834.

BLACKISTON, D. J., A. D. BRISCOE, AND M. R. WEISS. 2011. Colour vision and learning in the monarch butterfly, *Danaus plexippus* (Nymphalidae). *Journal of Experimental Biology* 214: 509-520.

BORGES, R. M., V. GOWDA, AND M. ZACHARIAS. 2003. Butterfly pollination and high-contrast visual signals in a low-density distylous plant. *Oecologia* 136: 571-573.

BOTES, C., S. D. JOHNSON, AND R. M. COWLING. 2008. Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering phenology. *Oikos* 117: 875-882.

BRISCOE, A. D., AND L. CHITTKA. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46: 471-510.

BROWN, M., C. T. DOWNS, AND S. D. JOHNSON. 2008. Sugar preferences of nectar feeding birds - a comparison of experimental techniques. *Journal of Avian Biology* 39: 479-483.

BROWN, M., C. T. DOWNS, AND S. D. JOHNSON. 2010. Concentration-dependent sugar preferences of the Malachite sunbird (*Nectarinia famosa*). *The Auk* 127: 151-155.

BROYLES, S. B., AND R. WYATT. 1993. The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *American Journal of Botany* 80: 41-44.

BURD, M. 1994. Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review* 60: 83-139.

BUTLER, H. C., AND S. D. JOHNSON. 2019 in press. Butterfly wing pollination in *Scadoxus* and other South African Amaryllidaceae. *Botanical Journal of the Linnaean Society*: Accepted pending minor revisions.

BUTYNSKI, T. M. 1990. Comparative ecology of blue monkeys (*Cercopithecus mitis*) in high- and low-density subpopulations. *Ecological Monographs* 60: 1-26.

CAMPBELL, D. R., A. JÜRGENS, AND S. D. JOHNSON. 2016. Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices. *New Phytologist* 210: 333-342.

CHALKER-SCOTT, L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* 70: 1-9.

CHAPMAN, C. A., AND S. E. RUSSO. 2007. Primate seed dispersal: linking behavioural ecology with forest community. *In* C. J. Campbell, A. F. Fuentes, K. C. MacKinnon, M. Panger, AND S. Bearder [eds.], Primates in perspective, 510-525. Oxford University Press, Oxford, UK.

CHAPMAN, C. A., L. J. CHAPMAN, M. CORDS, J. MWANGI GATHUA, A. GAUTIER-HION, J. E. LAMBERT, K. RODE, et al. 2002. The guenons: Diversity and adaptation in African monkeys. Kluwer Academic/Plenum Publishers, New York, USA.

CHARLESWORTH, D. 1989. Evolution of low female fertility in plants: pollen limitation, resource allocation and genetic load. *Trends in Ecology & Evolution* 4: 289-292.

CHAZDON, R. L. 1988. Sunflecks and their importance to forest understorey plants. *Advances in Ecological Research* 18: 1-63.

CHEUNG, A. Y. 1996. Pollen-pistil interactions during pollen-tube growth. *Trends in Plant Science* 1: 45-51.

CHITTKA, L., AND N. M. WASER. 1997. Why red flowers are not invisible to bees. *Israel Journal of Plant Sciences*. 45: 169-184.

CHORDS, M. 1986. Interspecific and intraspecific variation in diet of two forest guenons, *Cercopithecus ascanius* and *C. mitis*. *Journal of Animal Ecology* 55: 811-827.

CLARK, D. B., AND D. A. CLARK. 1987. Leaf production and the cost of reproduction in a tropical rain forest cycad, *Zamia skinneri*. *Journal of Ecology* 76: 1153-1163.

COLEMAN, B. T., AND R. A. HILL. 2014. Biogeographic variation in the diet and behaviour of *Cercopithecus mitis*. *Folia Primatologica* 85: 319-334.

CONNELL, J. H. 1971. On the role of natural enemies in preventing competitive exclusion in som marine mammals and in rain forest trees. *In* P. J. Boer AND G. R. Gradwell [eds.], Dynamics of Populations. Proceedings of the Advanced Study Institute on dynamics of numbers in population, Oosterbeck, September 7-18, 1970, 298-312. Centre for Agricultural Publishing and Documentation, Wageningen.

CONRAD, F. 2008. Molecular systematics, biogeography and dating of the tribe Haemantheae (Amaryllidaceae) and the phylogeography of *Clivia*. PhD thesis, University of Cape Town, Cape Town.

CONRAD, F., AND D. SNIJMAN. 2011. Systematics and Phylogeography of *Clivia*. *In* R. Fisher AND R. Dixon [eds.], Clivia Thirteen. *Clivia* Society, Pretoria, South Africa.

CONRAD, F., G. REEVES, AND J. P. ROURKE. 2003. Phylogenetic relationships of the recently discovered species - *Clivia mirabilis*. *South African Journal of Botany* 69: 204-206.

CONRAD, F., G. REEVES, D. A. SNIJMAN, AND T. A. J. HEDDERSON. 2006. Genetic relationships within the tribe Haemantheae (Amaryllidaceae) based on plastid DNA sequence data. *In* S. A. Ghazanfar AND H. J. Beentje [eds.], Taxonomy and ecology of African plants, their conservation and sustainable use, 73-83. Royal Botanic Gardens Kew, UK.

CORBERA, J., C. ALVAREZ-CROS, AND C. STEFANESCU. 2018. Evidence of butterfly wing pollination in the martagon lily *Lilium martagon* L. *Butlletí de la Institució Catalana d'Història Natural* 82: 117-120.

CORLETT, R. T., AND P. W. LUCAS. 1990. Alternative seed-handling strategies in primates: seed-spitting by long-tailed macaques (*Macaca fasciularis*). *Oecologia* 82: 166-171.

CROUCH, N. R., D. A. MULHOLLAND, T. L. POHL, AND E. NDLOVU. 2003. The ethnobotany and chemistry of the genus *Clivia* (Amaryllidaceae). *Journal of South African botany.* 69: 144-147.

CRUDEN, R. W. 1972. Pollinators in high-elevation ecosystems: relative effectiveness of birds and bees. *Science* 176: 1439-1440.

CRUDEN, R. W., AND S. M. HERMANN-PARKER. 1979. Butterfly pollination of *Caesalpinia pulcherrima,* with observations on a psychophilous syndrome. *Journal of Ecology* 67: 155-168.

CRUDEN, R. W., S. M. HERMANN, AND S. PETERSON. 1983. Patterns of nectar production and plant pollinator coevolution. *In* B. Bentley AND T. S. Elias [eds.], The biology of nectaries, 80-125. Columbia University Press, New York, USA.

CUTHILL, I. C., J. C. PARTRIDGE, A. T. D. BENNETT, S. C. CHURCH, N. S. HART, AND S. HUNT. 2000. Ultraviolet vision in birds. *Advances in the Study of Behavior* 29: 159-214.

DE WAAL, C. 2010. Reproductive ecology of bird-pollinated *Babiana* (Iridaceae): floral variation, mating patterns and genetic diversity. Master's thesis. University of Toronto, Toronto, Canada.

DE WAAL, C., S. C. H. BARRETT, AND B. ANDERSON. 2012. The effect of mammalian herbivory on inflorescence architecture in the ornithophilous *Babiana* (Iridaceae): implications for the evolution of a bird perch. *American Journal of Botany* 99: 1096-1103.

DE WAAL, C., B. ANDERSON, AND S. C. H. BARRETT. 2012. The natural history of pollination and mating in bird-pollinated *Babiana* (Iridaceae). *Annals of Botany* 109: 667-679.

DENSLOW, J. S. 1987. Topical rainforest gaps and tree species diveristy. *Annual Review of Ecology and Systematics* 18: 431-451.

DOBSON, H. E. M. 2006. Relationship between floral fragrance composition and type of pollinator. *In* N. Dudareva AND E. Pichersky [eds.], Biology of floral scents, 147-198. Taylor & Francis, Boca Raton, USA.

DOBZHANSKY, T. 1951. Genetics and the origin of species, 3rd ed. Columbia University Press, New York, USA.

DUNCAN, G. D. 1999. Grow *Clivias*. 3rd ed. National Botanical Institute, Cape Town, South Africa.

DUTRILLAUX, B., M. MULERIS, AND J. C. COUTURIER. 1988. Chromosomal evolution of Cercopithecinae. *In* A. Gautier-Hion, F. Bourliere, J.-P. Gautier, AND J. Kingdon [eds.], A primate radiation: Evolutionary biology of African guenons, 150-159. Cambridge Univeristy Press, Cambridge, UK.

EDWARDS, J., AND J. R. JORDAN. 1992. Reversible anther opening in *Lilium philadelphicum* (Liliaceae): a possible means of enhancing male fitness. *American Journal of Botany* 79: 144-148.

EGUCHI, E., K. WATANABE, T. HARIYAMA, AND K. YAMAMMOTO. 1982. A comparasion of electrophysiologically determined spectral responses in 35 species of Lepidoptera. *Journal of Insect Physiology* 28: 675-682.

ELISENS, W. J., AND C. E. FREEMAN. 1988. Floral nectar sugar composition and pollinator type among new world genera in tribe Antirrhineae (Scrophulariaceae). *American Journal of Botany* 75: 971-978.

ENDLER, J. A., AND P. W. MIELKE. 2005. Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society* 86: 405-431.

EPPS, M. J., S. E. ALLISON, AND L. M. WOLFE. 2015. Reproduction in flame azalea (*Rhododendron calendulaceum*, Ericaceae): a rare case of insect wing pollination. *The American Naturalist* 186: 294-301.

ESTRADA, A., P. A. GARBER, A. B. RYLANDS, C. ROOS, E. FERNANDEZ-DUQUE, A. DI FIORE, K. A.-I. NEKARIS, et al. 2017. Impending extinction crisis of the world's primates: Why primates matter. *Science Advances* 3: e1600946.

FAEGRI, K., AND L. VAN DER PIJL. 1979. The Principles of Pollination Ecology. Pergamon, Oxford, UK.

FARNSWORTH, E. 2000. The ecology and physiology of viviparous and recalcitrant seeds. *Annual Review of Ecology and Systematics* 31: 107-138.

FLEAGLE, J. G. 1999. Primate adaptation and evolution. Academic Press, New York, USA.

FOX, R. M. 1966. Forelegs of butterflies. *Journal of Research on the Lepidoptera* 5: 1-12.

FREEMAN, C. E., W. H. REID, J. E. BECVAR, AND R. SCOGIN. 1984. Similarity and apparent convergence in the nectar-sugar composition of some hummingbird-pollinated flowers. *Botanical Gazette* 145: 132-135.

FULTON, M., AND S. A. HODGES. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society of London, Series B, Biological Science* 266: 2247-2252.

GARBER, P. A., AND J. E. LAMBERT. 1998. Primates as seed dispersers: ecological processes and directions for future research. *American Journal of Primatology* 45: 3-8.

GAUTIER-HION, A., J. M. DUPLANTIER, R. QURIS, F. FEER, C. SOURD, J. P. DECOUX, G. DUBOST, et al. 1985. Fruit characteristics as a basis of fruit choice and seed dispersal in a tropical forest vertebrate community. *Oecologia* 65: 324-337.

GEERTS, S., AND A. PAUW. 2007. A malachite sunbird pollination guild in the Cape flora, with implications for the endangered *Brunsvigia litoralis*. *South African Journal of Botany* 73: 289-289.

GEERTS, S., AND A. PAUW. 2009. Hyper-specialization for long-billed bird pollination in a guild of South African plants: the malachite sunbird pollination syndrome. *South African Journal of Botany* 75: 699-706.

GEERTS, S., AND A. PAUW. 2012. The cost of being specialized: pollinator limitation in the endangered geophyte *Brunsvigia litoralis* (Amaryllidaceae) in the Cape Floristic Region of South Africa. *South African Journal of Botany* 78: 159-164.

GÉNIN, F., A. YOKWANA, N. KOM, S. COUETTE, T. DIEULEVEUT, S. D. NASH, AND J. C. MASTERS. 2016. A new galago species for South Africa (Primates: Strepsirhini: Galagidae). *African Zoology* 51: 135-143.

GIBBS, P. E. 2014. Late acting self incompatibility - the pariah breeding system in flowering plants. *New Phytologist* 203: 717-734.

GIBBS, P. E., AND M. BIANCHI. 1993. Post-pollination events in species of *Chorisia* (Bombacaceae) and *Tabebuia* (Bignoniaceae) with late-acting self-incompatibility. *Botanica Acta* 106: 64-71.

GIBBS, P. E., AND M. A. BIANCHI. 1999. Does late-acting self-incompatibility (LSI) show family clustering? Two more species of Bignoniaceae with LSI: *Dolichandra cynanchoides* and *Tabebuia nodosa*. *Annals of Botany* 84: 449-457.

GIURFA, M., A. DAFNI, AND P. R. NEAL. 1999. Floral symmetry and its role in plant-pollinator systems. *International Journal of Plant Sciences* 160: S41-S50.

GOLDSMITH, T. H., AND K. M. GOLDSMITH. 1979. Discrimination of colors by the Black-chinned Hummingbird (*Archilochus alexandri*). *Journal of Comparative Physiology A (Sensory, Neural, and Behavioral Physiology)* 230: 209-220.

GOMEZ, L. G., D. C. HOUSTON, P. COTTON, AND A. TYE. 1994. The role of greater yellow-headed vultures, *Cathartes melambrotus*, as scavengers in Neotropical forest. *Ibis* 136: 193-196.

GOULD, E., AND E. S. VRBA. 1982. Exaptation-a missing term in the science of form. *Paleobiology* 8: 4-15.

GOULSON, D., AND J. S. CORY. 1993. Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecological Entomology* 18: 315-320.

GOULSON, D., J. C. STOUT, AND S. A. HAWSON. 1997. Can flower constancy in nectaring butterflies be explained by Darwin's interference hypothesis? *Oecologia* 112: 225-231.

GOULSON, D., S. A. HAWSON, AND J. C. STOUT. 1998. Foraging bumblebees avoid flowers already visited by conspecifics or by other bumblebee species. *Animal Behaviour* 55: 199-206.

GOULSON, D., J. W. CHAPMAN, AND W. O. H. HUGHES. 2001. Discrimination of unrewarding flowers by bees; direct detection of rewards and use of repellent scent marks. *Journal of Insect Behavior* 14: 669-678.

GRANT, K. A. 1966. A hypothesis concerning the prevalence of red coloration in California hummingbird flowers. *American Naturalist* 100: 85-97.

GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82-97.

------. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences, USA* 91: 3-10.

GRANT, V., AND K. A. GRANT. 1965. Flower pollination in the Phlox family. Columbia University Press, New York, USA.

GÜBITZ, T., M. E. HOBALLAH, A. DELL' OLIVO, AND C. KUHLEMEIR. 2009. *Petunia* as a model system for the genetics and evolution of pollination syndromes. *In* T. Gerats AND J. Strommer [eds.], Petunia, 29-49. Springer, New York, USA.

HAMILTON, A. C., AND D. TAYLOR. 1992. History of climate and forests in tropical Africa during the last 8 million years. *In* N. Myers [ed.], Tropical forests and climate, 65-78. Springer Netherlands, Dordrecht, Netherlands.

HAO, Y. Q., X. F. ZHAO, D. Y. SHE, B. XU, D. Y. ZHANG, AND W. J. LIAO. 2012. The role of late-acting self-incompatibility and early-acting inbreeding depression in governing female fertility in monkshood, *Aconitum kusnezoffii*. *PLOS ONE* 7: 1-7.

HARDER, L. D., AND M. A. AIZEN. 2010. Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society Series B* 365: 529-543.

HARDER, L. D., S. C. H. BARRETT, AND W. W. COLE. 2000. The mating consequences of sexual segregation within inflorescences of flowering plants. *Proceedings of the Royal Society of London, Series B, Biological Science* 267: 315-320.

HAUS, T., E. AKOM, B. AGWANDA, M. HOFREITER, C. ROOS, AND D. ZINNER. 2013. Mitochondrial diversity and distribution of african Green Monkeys (*Chlorocebus* Gray, 1870). *American Journal of Primatology* 75: 350-360.

HAVERKAMP, A., X. LI, B. S. HANSSON, I. T. BALDWIN, M. KNADEN, AND F. YON. 2019. Flower movement balances pollinator needs and pollen protection. *Ecology* 100: e02553.

HENDRIX, S. D. 1988. Herbivory and its impact on plant reproduction. *In* J. Lovett Doust AND L. D. Lesley [eds.], Plant Reproductive Ecology, 246-263. Academic Press, Orlando, Florida, USA.

HENNING, G. A., S. F. HENNING, J. G. JOANNOU, AND S. E. WOODHALL. 1997. Living butterflies of southern Africa. Umdaus Press, Pretoria, South Africa.

HIROTA, S. K., N. MIKI, A. A. YASUMOTO, AND T. YAHARA. 2019. UV bullseye contrast of *Hemerocallis* flowers attracts hawkmoths but not swallowtail butterflies. *Ecology and Evolution* 9: 52-64.

HIROTA, S. K., K. NITTA, Y. KIM, N. KAWAKUBO, A. A. YASUMOTO, AND T. YAHARA. 2012. Relative role of flower colour and scent on pollinator attraction: Experimental tests using F1 and F2 hybrids of daylily and nightlily. *PLOS ONE* 7: e39010.

HONDA, K., H. ÔMURA, AND N. HAYASHI. 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower-visiting by Cabbage butterfly, *Pieris rapae*. *Journal of Chemical Ecology* 24: 2167-2180.

HOWE, H. F., AND J. SMALLWOOD. 1982. Ecology of seed dispersal. *Annual Review of Ecology and Systematics* 13: 201-228.

HOWE, H. F., AND M. N. MIRITI. 2004. When seed dispersal matters. *BioScience* 54: 651-660.

HOWELL, G., AND N. PRAKASH. 1990. Embryology and reproductive ecology of the Darling lily, *Crinum flaccidum* Herbert. *Australian Journal of Botany* 38: 433-444.

HUBBELL, S. P., R. B. FOSTER, S. T. O'BRIEN, K. E. HARMS, R. CONDIT, B. WECHSLER, S. J. WRIGHT, AND S. LOO DE LAO. 1999. Light-gap disturbances, recruitment limitation and tree diversity in a neotropical forest. *Science* 283: 554-557.

ILSE, D. 1928. Uber den Farbensinn der Tagfalter. *Z. f. vergl. Physiologie Bd.* 8: 658-692.

ILSE, D., AND V. G. VAIDYA. 1956. Spontaneous feeding response to colours in *Papilio demoleus* L. *Proceedings of the Indian Academy of Science, Section B, Plant Science* 43: 23-31.

JANZEN, D. H. 1970. Herbivores and the number of tree species in tropical forests. *American Naturalist* 104: 501-528.

JOHNSON, S. D. 1996a. Bird pollination in South African species of *Satyrium* (Orchidaceae). *Plant Systematics and Evolution* 203: 91-98.

JOHNSON, S. D. 1996b. Pollination, adaptation and speciation models in the Cape flora of South Africa. *Taxon* 45: 59-66.

JOHNSON, S. D. 2006. Pollinator-driven speciation in plants. *In* L. D. Harder AND S. C. H. Barrett [eds.], The ecology and evolution of flowers 295-310. Oxford University Press, Oxford, UK.

JOHNSON, S. D., AND W. J. BOND. 1994. Red flowers and butterfly pollination in the fynbos of South Africa. *In* M. Arianoutsou AND R. H. Groves [eds.], Plant-animal interactions in Mediterranean-type ecosystems, 137-148. Kluwer Academic Publishers, Dordrecht, Netherlands.

JOHNSON, S. D., AND S. W. NICOLSON. 2008. Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biology Letters* 4: 49-52.

JOHNSON, S. D., AND P. WESTER. 2017. Stefan Vogel's analysis of floral syndromes in the South African flora: An appraisal based on 60 years of pollination studies. *Flora* 232: 200-206.

JOHNSON, S. D., A. L. HARGREAVES, AND M. BROWN. 2006. Dark bitter-tasting nectar functions as a filter of flower visitors in a bird-pollinated plant. *Ecology* 87: 2709-2716.

JOHNSON, S. D., H. C. BUTLER, AND A. ROBERTSON. 2019. Breeding systems in *Cyrtanthus* (Amaryllidaceae): variation in self-sterility and potential for ovule discounting. *Plant Biology* doi: 10.1111/plb.13025.

KANDORI, I., AND N. OHSAKI. 1996. The learning abilities of the white cabbage butterfly, *Pieris rapae*, foraging for flowers. *Researches on Population Ecology* 38: 111-117.

------. 1998. Effect of experience on foraging behavior towards artificial nectar guide in the cabbage butterfly, *Pieris rapae crucivora* (Lepidoptera: Pieridae). *Applied Entomology and Zoology* 33: 35-42.

KANDORI, I., AND T. YAMAKI. 2012. Reward and non-reward learning of flower colours in the butterfly *Byasa alcinous* (Lepidoptera: Papilionidae). *Naturwissenschaften* 99: 705–713.

KANDORI, I., T. YAMAKI, S.-I. OKUYAMA, N. SAKAMOTO, AND T. YOKOI. 2009. Interspecific and intersexual learning rate differences in four butterfly species. *Journal of Experimental Biology* 212: 3810-3816.

KAPLIN, B. A. 2001. Ranging behavior of two species of guenons (*Cercopithecus lhoesti* and *C. mitis doggetti*) in the Nyungwe Forest Reserve, Rwanda. *International Journal of Primatology* 22: 521-548.

KAPLIN, B. A., AND T. C. MOERMOND. 1998. Variation in seed handling by two species of forest monkeys in Rwanda. *American Journal of Primatology* 45: 83-101.

KERNER VON MARILAUN, A. 1902. The Natural History of Plants. Blackie, London, UK.

KIEPIEL, I., AND S. D. JOHNSON. 2019. Spit it out: Monkeys disperse the unorthodox and toxic seeds of *Clivia miniata* (Amaryllidaceae). *Biotropica* 51: 619-625.

KINGSOLVER, J. G., AND T. L. DANIEL. 1979. On the mechanics and energetics of nectar feeding butterflies. *Journal of Theoretical Biology* 76: 167-179.

KINOSHITA, M., N. SHIMADA, AND K. ARIKAWA. 1999. Colour vision of the foraging swallotail butterfly *Papilio xunthus*. *The Journal of Experimental Biology* 202: 95-102.

KINOSHITA, M., F. J. STEWART, AND H. ÔMURA. 2017. Multisensory integration in Lepidoptera: Insights into flower-visitor interactions. *BioEssays* 39: 1600086.

KNUDSEN, J. T., AND L. TOLLSTEN. 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. *Botanical Journal of the Linnean Society* 113: 263-284.

KNUDSEN, J. T., R. ERIKSSON, J. GERSHENZON, AND B. STAHL. 2006. Diversity and distribution of floral scent. *Botanical Review* 72: 1-120.

KNUDSEN, J. T., L. TOLLSTEN, I. GROTH, G. BERGSTROM, AND R. A. RAGUSO. 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society* 146: 191-199.

KNÜTTEL, H., AND K. FIEDLER. 2001. Host-plant derived variation in ultraviolet wing patterns influences mate selection by male butterflies. *Journal of Experimental Biology*. 204: 2447-2459.

KOCIKOVA, L., D. MIKLISOVA, A. CANADY, AND L. PANIGAJ. 2012. Is colour an important factor influencing the behaviour of butterflies (Lepidoptera: Hesperioidea, Papilionoidea)? *European Joural of Entomology* 109: 403-410.

KOOPOWITZ, H. 2002. *Clivias*. Timber Press, Portland, USA.

KOSHITAKA, H., M. KINOSHITA, M. VOROBYEV, AND K. ARIKAWA. 2008. Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proceedings of the Royal Society B* 275: 947-954.

LAMBERT, J. E. 1998. Primate frugivory in Kibale National Park, Uganda, and its implications for human use of forest resources. *African Journal of Ecology* 36: 234-240.

LAMBERT, J. E. 1999. Seed handling in chimpanzees (*Pan troglodytes*) and redtail monkeys (*Cercopithecus ascanius*): implications for understanding hominid and cercopithecine fruit-processing stratergies and seed dispersal. *American Journal of Physical Anthropology* 109: 365-386.

LAMBERT, J. E. 2001. Red-tailed guenons (*Cercophithecus ascanius*) and *Strychnos mitis:* evidence for plant benefits beyond seed dispersal. *International Journal of Primatology* 22: 189-201.

------. 2002. Exploring the link between animal frugivory and plant stratergies: the case of primate fruit processing and post-dispesal seed fate. *In* D. J. Levey, W. R. Silva, AND M. Galetti [eds.], Seed dispersal and frugivory: ecology, evolution and conservation. Third International Symposium-Workshop on Frugivores and Seed Dispersal, Sao Pedro, Brazil, 6-11 August 2000, 365-379. CABI Publishing, Wallingford, UK.

LAWES, M. J. 1990a. The Distribution of the Samango Monkey (*Cercopithecus mitis erythrarchus* Peters, 1852 and *Cercopithecus mitis labiatus* I. Geoffroy, 1843) and forest history in southern Africa. *Journal of Biogeography* 17: 669-680.

------. 1990b. Diet and feeding behavior of samango monkeys (*Cercopithecus mitis labiatus*) in Ngoye Forest, South Africa. *Folia Primatologica* 54: 57-69.

------. 1991. Diet of samango monkeys (*Cercopithecus mitis erythrarchus*) in the Cape Vidal Dune Forest, South Africa. *Journal of Zoology* 244: 149-173.

------. 1992. Estimates of population density and correlates of the status of the samango monkey *Cercopithecus mitis* in Natal, South Africa. *Biological Conservation* 60: 197-210.

LAWES, M. J., S. P. HENZI, AND M. R. PERRIN. 1990. Diet and feeding-behavior of samango monkeys (*Cercopithecus mitis labiatus*) in Ngoye Forest, South Africa. *Folia Primatologica* 54: 57-69.

LEWIS, A. C. 1986. Memory constraints and flower choice in *Pieriis rapae*. *Science* 232: 863-865.

LEWIS, A. C. 1989. Flower visit consistency in *Pieris rapae*, the cabbage butterfly. *Journal of Animal Ecology* 58: 1-13.

LEWIS, A. C., AND G. A. LIPANI. 1990. Learning and flower use in butterflies: Hypotheses from honey bees. *In* E. A. Bernays [ed.], Insect-plant interactions, 95-110. CRC Press, Boca Raton, USA.

LINDEN, B., J. LINDEN, F. FISCHER, AND K. E. LINSENMAIR. 2015. Seed dispersal by South Africa's only forest-dwelling guenon, the samango monkey (*Cercopithecus mitus*). *African Journal of Wildlife Research* 45: 88-99.

LIPOW, S. R., AND R. WYATT. 2000. Single gene control of post-zygotic self-incompatibiltiy in poke milkweed, *Asclepias exaltata* L *Genetics* 154: 893-907.

LOTZ, C. N., AND S. W. NICHOLSON. 1996. Sugar preferences of a nectarivorous passerine bird, the Lesser Double-collared Sunbird (*Nectarinia chalybea*). *Functional Ecology* 10: 360-365.

MANNING, J. 2005. Pollination in *Clivia*. *In* R. Dixon, C. Felbert, AND J. van der Linde [eds.], *Clivia* Seven, 17-22. *Clivia* Society, Cape Town, South Africa.

MARTINEZ DEL RIO, C. 1990. Dietry, phylogenetic and ecological correlates of intestinal sucrase and maltase activity in birds. *Physiological Zoology* 63: 987-1011.

MEEROW, A. W., AND D. A. SNIJMAN. 1998. Amaryllidaceae *In* K. Kubitzki [ed.], Families and genera of vascular plants vol. 3, 83-110. Springer Verlag, Berlin, Germany.

MEESTER, J. A. J., I. L. RAUTENBACH, N. J. DIPPENAAR, AND C. M. BAKER. 1986. Classification of southern African mammals. Transvaal Museum Monograph No. 5. Transvaal Museum, Pretoria, South Africa.

MERY, F., AND T. J. KAWECKI. 2004. An operating cost of learning in *Drosophila melanogaster*. *Animal Behaviour* 68: 589-598.

MIURA, S., M. YASUDA, AND L. C. RATNAM. 1997. Who steals the fruit? Monitoring frugivory of mammals in a tropical rain-forest. *The Malayan Nature Journal* 50: 183-193.

NASRALLAH, J. B., J. C. STEIN, M. K. KANDASAMY, AND M. E. NASRALLAH. 1994. Signaling the arrest of pollen tube development in self-incompatible plants. *Science* 266: 1505-1508.

NELSON, L. S., R. D. SHIH, AND M. J. BALICK. 2007. Handbook of poisonous and injurious plants. Second Editon ed. The New York Botanical Garden, Springer, New York, USA.

NEVITT, G. A., R. R. VEIT, AND P. KAREIVA. 1995. Dimethyl sulphide as a foraging cue for Antarctic Procellariiform seabirds. *Nature.* 376: 680-682.

NORCONK, M. A., B. W. GRAFTON, AND N. L. CONKLIN-BRITTAIN. 1998. Seed dispersal by neotropical seed predators. *American Journal of Primatology* 45: 103-126.

NYIRAMANA, A., I. MENDOZA, B. A. KAPLIN, AND P. M. FORGET. 2011. Evidence for seed dispersal by rodents in tropical montane forest in Africa. *Biotropica* 43: 654-657.

ÔMURA, H., AND K. HONDA. 2005. Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* 142: 588-596.

ÔMURA, H., K. HONDA, AND N. HAYASHI. 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. *Journal of Chemical Ecology* 25: 1895-1906.

ÔMURA, H., K. HONDA, A. NAKAGAWA, AND N. HAYASHI. 1999. The role of floral scent of the cherry tree, *Prunus yedoensis*, in the foraging behavior of *Leuhdorfia japonica* (Lepidoptera: Papilionidae). *Applied Entomology and Zoology* 34: 309-313.

PAUW, A. 2019. A bird's-eye view of pollination: Biotic interactions as drivers of adaptation and community change. *Annual Review of Ecology, Evolution and Systematics* 50: 477-502.

PEITSCH, D., A. FIETZ, H. HERTEL, J. DE SOUZA, D. F. VENTURA, AND R. MENZEL. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. *Journal of Comparative Physiology A (Sensory, Neural, and Behavioral Physiology)* 170: 23-40.

POHL, N. B., J. VAN WYK, AND D. R. CAMPBELL. 2011. Butterflies show flower colour preferences but not constancy in foraging at four plant species. *Ecological Entomology* 36: 290-300.

PRIMACK, R. B. 1980. Variation in the phenology of natural populations of montane shrubs in New Zealand. *Journal of Ecology* 68: 849-862.

PRIMACK, R. B. 1985. Longevity of individual flowers. *Annual Review of Ecology and Systematics* 16: 15-37.

PROCTOR, M., P. YEO, AND A. LACK. 1996. The Natural History of Pollination. Collins, London, UK.

RAGUSO, R. A., AND E. PICHERSKY. 1995. Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): recent evolution of floral scent and moth pollination. *Plant Systematics and Evolution* 194: 55-67.

RAN, Y., K. R. W. HAMMETT, AND B. G. MURRAY. 2001. Phylogenetic analysis and karyotype evolution in the genus *Clivia* (Amaryllidaceae). *Annals of botany* 87: 823-830.

RAVEN, P. H. 1972. Why are bird-visited flowers predominantly red. *Evolution* 26: 674.

RODGER, J. G., M. VAN KLEUNEN, AND S. D. JOHNSON. 2013. Pollinators mates and Allee effects: the importance of self-pollination for fecundity in an invasive lily. *Functional Ecology* 27: 1023-1033.

RODRIGUES, D., B. W. GOODNER, AND M. R. WEISS. 2010. Reversal learning and risk-averse foraging behavior in the monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae). *Ethology* 116: 270-280.

RODRIGUEZ-GIRONES, M. A., AND L. SANTAMARIA. 2004. Why are so many bird flowers red? *Plos Biology* 2: 1515-1519.

ROURKE, J. P. 2002. *Clivia mirabilis* (Amaryllidaceae: Haemantheae) a new species from Northern Cape, South Africa. *Bothalia* 32: 1-7.

RUSTERHOLZ, H.-P., AND A. ERHARDT. 1997. Preferences for nectar sugars in the peacock butterfly, *Inachis io*. *Ecological Entomology* 22: 220-224.

SAGE, T. L., AND E. G. WILLIAMS. 1991. Self-incompatibility in *Asclepias*. *Plant Cell Incompatibility Newsletter* 23: 55-57.

SAGE, T. L., M. V. PRICE, AND N. M. WASER. 2006. Self-sterility in *Ipomopsis aggregata* (Polemoniaceae) is due to prezygotic ovule degeneration. *American Journal of Botany* 93: 254-262.

SAGE, T. L., F. STRUMAS, W. W. COLE, AND S. C. H. BARRETT. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* 86: 855-870.

SCHERER, C., AND G. KOLB. 1987a. Behavioral experiments on the visual processing of color stimuli in *Pieris brassicae* L. (Lepidoptera). *Journal of Comparative Physiology A (Sensory, Neural, and Behavioral Physiology)* 160: 645-656.

------. 1987b. The influence of color stimuli on visually controlled behavior in *Aglais urticae* L. and *Pararge aegeria* L. (Lepidoptera). *Journal of Comparative Physiology A (Sensory, Neural, and Behavioral Physiology)* 161: 891-898.

SEAVEY, S. R., AND K. S. BAWA. 1986. Late-acting self-incompatibility in angiosperms. *Botanical Review* 52: 195-219.

SEAVEY, S. R., AND S. K. CARTER. 1994. Self-sterility in *Epilobium obcordatum* (Onagraceae). *American Journal of Botany* 81: 331-338.

SEUFERT, V., B. LINDEN, AND F. FISCHER. 2010. Revealing secondary seed removers: results from camera trapping. *African Journal of Ecology* 48: 914-922.

SILBERGLIED, R. E. 1984. Visual communication and sexual selection among butterflies. *In* R. I. Vane-Wright AND P. R. Ackery [eds.], The Biology of Butterflies, 207-233. Academic Press, London, UK.

SIMPSON, B. B., AND J. L. NEFF. 1983. Evolution and diversity of floral rewards. *In* C. E. Jones AND R. J. Little [eds.], Handbook of experimental pollination biology, 142-159. Van Nostrand Reinhold., New York, USA.

SNELL-ROOD, E. C., G. DAVIDOWITZ, AND D. R. PAPAJ. 2011. Reproductive tradeoffs of learning in a butterfly. *Behavioral Ecology* 22: 291-302.

SNIJMAN, D. A., AND H. P. LINDER. 1996. Phylogenetic relationships, seed characters, and dispersal system evolution in Amaryllideae (Amaryllidaceae). *Annals of the Missouri Botanical Garden* 83: 362-386.

SONNE, J., T. B. ZANATA, A. M. M. GONZALEZ, N. L. C. TORRES, J. FJELDSA, R. K. COLWELL, B. A. TINOCO, C. RAHBEK, AND B. DALSGAARD. 2019. The distributions of morphologically specialized hummingbirds coincide with floral trait matching across an Andean elevational gradient. *Biotropica* 51**:** 205-218.

SPIES, P., AND J. J. SPIES. 2018. *Clivia* taxonomy revisited, using DNA barcode regions. *Acta Horticulturae* 1201: 503-514.

SPIES, P., J. P. GROBLER, AND J. J. SPIES. 2011. A review of phylogenetic relationships in the genus *Clivia*. *Philosophical Transactions in Genetics* 1: 168-207.

STEBBINS, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307-326.

STEBBINS, G. L., JR. 1950. Variation and evolution in plants. Columbia University Press, New York, USA.

STEENHUISEN, S.-L., R. A. RAGUSO, AND S. D. JOHNSON. 2012. Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function *Phytochemistry.* 84: 78-87.

STILES, F. G. 1971. Time, energy, and territoriality of the Anna hummingbird (*Calypte anna*). *Science* 173: 818-821.

STILES, F. G. 1976. Taste preferences, color preferences, and flower choice in hummingbirds. *Condor* 78: 10-26.

STOUT, J. C., D. GOULSON, AND J. A. ALLEN. 1998. Repellent scent-marking of flowers by a guild of foraging bumblebees (*Bombus* spp.). *Behavioral Ecology and Sociobiology* 43: 317-326.

STRAUSS, S. Y., AND A. R. ZANGREL. 2002. Plant–insect interactions in terrestrial ecosystems. *In* C. M. Herrera AND O. Pellmyr [eds.], Plant–animal interactions. An evolutionary approach, 77-106. Blackwell Science, Oxford, UK.

STREHER, N. S., E. GUERRA, R. LÜDTKE, J. SEMIR, AND J. H. A. DUTILH. 2018. Self-incompatibility in *Habranthus gracilifolius* (Amaryllidaceae): pre- and post-pollination barriers. *Brazilian Journal of Botany* 41: 375-384.

SWANEVELDER, D., AND R. C. FISHER. 2009. *Clivia*: Nature and nurture. Briza Publications, Pretoria, South Africa.

SWANEVELDER, Z. H., J. T. TRUTER, AND A. E. VAN WYK. 2006. A natural hybrid in the genus *Clivia*. *Bothalia* 36: 77-80.

SWIHART, C. A., AND S. L. SWIHART. 1970. Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius* Linn. *Animal Behaviour* 18: 60-64.

SWIHART, S. L. 1970. The neural basis of colour vision in the butterfly, *Papilio troilus*. *Journal of Insect Physiology* 16: 1623-1636.

TAKEDA, K. 1961. The nature of impulses of single tarsal chemoreceptors in the butterfly *Vanessa indica*. *Journal of Cellular and Comparative Physiology* 58: 233-245.

TOPAZZINI, A., M. MAZZA, AND P. PELOSI. 1990. Electroantennogram responses of five Lepidoptera species to 26 general odourants. *Journal of Insect Physiology* 36: 619-624.

TRIPP, E. A., AND P. S. MANOS. 2008. Is floral specialization and evolutionary dead end? Pollination system transitions in *Ruellia* (Acanthaceae). *Evolution; International Journal of Organic Evolution* 62: 1712-1737.

USHIMARU, A., AND F. HYODO. 2005. Why do bilaterally symmetrical flowers orient vertically? Flower orientation influences pollinator landing behaviour. *Evolutionary Ecology Research* 7: 252-260.

USHIMARU, A., D. KAWASE, AND A. IMAMURA. 2006. Flowers adaptively face down-slope in 10 forest-floor herbs. *Functional Ecology* 20: 585-591.

VAIDYA, V. G. 1969. Form perception in *Papilio demoleus L.* (Papilionidae, Lepidoptera). *Behaviour* 33: 212-221.

VAN DER MERWE, L., H. ROBBERTSE, AND B. DE KOCK. 2005. Cultivation of *Clivias*. *Clivia* Society, Pretoria, South Africa.

VAN DER PIJL, L. 1982. Principles of dispersal in higher plants. Springer-Verlag, Berlin, Germany.

VAN WYK, B.-E., B. VAN OUDTSHOORN, AND N. GERICKE. 2009. Medicinal plants of South Africa. Briza Publications, Pretoria, South Africa.

VARASSIN, I. G., J. R. TRIGO, AND M. SAZIMA. 2001. The role of nectar production, flower pigments and odour in the pollination of four species of *Passiflora* (Passifloraceae) in south-eastern Brazil. *Botanical Journal of the Linnean Society* 136: 139-152.

VAUGHTON, G., M. RAMSEY, AND S. D. JOHNSON. 2010. Pollination and late-acting self-incompatibility in *Cyrtanthus breviflorus* (Amaryllidaceae): implications for seed production. *Annals of Botany* 106: 547-555.

VILADOMAT, F., J. BASTIDA, C. CODINA, J. J. NAIR, AND W. E. CAMPBELL. 1997. Alkaloids of the South African Amaryllidaceae. *Recent Research Developments in Phytochemistry* 1: 131-171.

WASER, N. M., AND M. V. PRICE. 1991. Reproductive costs of self-pollination in *Ipomopsis aggregata* (Polemoniaceae): are ovules usurped? *American Journal of Botany* 78: 1036-1043.

WEISS, M. R. 1995. Associative colour learning in a nymphalid butterfly. *Ecological Entomology* 20: 298-301.

------. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Animal Behaviour* 53: 1043-1052.

WEISS, M. R. 2001. Vison and learning in some neglected pollinators: beetles, flies moths and butterflies. *In* L. Chittka AND J. D. Thomson [eds.], Cognitive ecology of pollination: animal behaviour and floral evolution, 171-190. Cambridge University Press, Cambridge, UK.

WEISS, M. R., AND D. R. PAPAJ. 2003. Colour learning in two behavioural contexts: how much can a butterfly keep in mind? *Animal Behaviour* 65: 425-434.

WESTER, P., AND R. CLAßEN-BOCKHOFF. 2006. Bird pollination in South African *Salvia* species. *Flora* 201: 396-406.

WESTERKAMP, C. 1990. Bird-flowers: hovering versus perching exploitation. *Botanica Acta* 103: 366-371.

WHITTALL, J. B., AND S. A. HODGES. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 477: 706-709.

WIKLUND, C., T. ERICSON, AND H. LUNDBERG. 1979. The wood white butterfly *Leptidea sinapis* and its nectar plants: a case of mutualism. *Oikos* 33: 358-362.

WILSON, M. F. 1993. Dispersal mode, seed shadows, and colonization patterns. *Vegetatio* 107/108: 261-280.

WILTSCHKO, R. 1996. The function of olfactory input in pigeon orientation: does it improve navigational information or play another role? *Journal of Experimental Biology*. 199: 113-119.

WINK, M., AND B.-E. VAN WYK. 2008. Mind-altering and poisonous plants of the world. Briza Publications, Pretoria, South Africa.

WOLFHEIM, J. H. 1983. Primates of the world: Distribution, abundance, and conservation. University of Washinton Press, Seattle, USA.

WOODHALL, S. E. 2005. Field guide to butterflies of South Africa. Struik Nature, Cape Town, South Africa.

YON, F., D. KESSLER, Y. JOO, L. CORTÉS LLORCA, S.-G. KIM, AND I. T. BALDWIN. 2017. Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*. *Journal of Integrative Plant Biology* 59: 180-189.

MICROSATELLITE MARKERS IN *CLIVIA*: PRELIMINARY RESEARCH

The aims of this research were: (1) develop and test novel SSR markers for the genus, (2) to use these SSR markers to assess to assess phylogeographical and population genetic structure among populations of *C. gardenii* and *C. robusta* and (3), and to study outcrossing rates in *C. miniata* and *C. gardenii*. This research was initiated at the start of 2015 at a time when the relationship between *C. robusta*, *C. gardenii* and *C. miniata* was unresolved (Spies, Grobler, and Spies, 2011). The application of SSR's (simple sequence repeats or microsatellites) began with the taxonomic debate surrounding the "swamp gardenii" *C. robusta* and was prompted by the publication of 14 *Clivia* microsatellites (Gao et al., 2012). Reports of haplotype sharing between *C. gardenii* and *C. robusta* (Conrad and Snijman, 2011), and the suggestion that *C. robusta* was simply a "robust" form of *C. gardenii* (Rourke, 2012), sparked an enquiry assessing the phylogenetic relationships between these two taxa. In order to assess phylogeographical and genetic relationships among populations of *C. gardenii* and *C. robusta* as was done successfully for *Streptocarpus* Lindl. in the mosaic of forest patches in eastern southern Africa (Hughes et al., 2004; Hughes et al., 2005), a study was undertaken using published SSR markers (Gao et al., 2012). What follows is a brief discussion on this preliminary SSR work in *Clivia*.

Leaf material was obtained from forests in the Pondoland Centre of Endemism (PC) and south eastern regions of KZN Province, South Africa. It is likely that extensive ongoing illegal collection of plants has contributed to the exceptional rarity of *C.* robusta and relics of populations were frequently encountered. Isolated individuals were frequently found in cliffs or hidden in trees, and extensive plant removal was widespread in even in protected areas. Some consider the southernmost distribution of *C. gardenii* to end at Port Edward (Felbert, 2003), whilst suggestions have been made that *C. robusta* occurs only in the Pondoland Centre of Endemism (south of Port Edward) as far as Lusikisiki (Dixon, 2005). Sampling of leaf material in this range was extensively covered. Leaf material was only collected when plants were in flower. Sampling expeditions extended as far as south as Dwesa in the Eastern Cape and as far north as Harburg in KZN. Leaf material was collected for a total of 10 populations; five in KZN and five in the Eastern Cape. Leaf samples were taken from newly emerging leaves and immediately placed into individually labelled leaf envelopes which were in turn placed into double sealed air tight plastic bags containing silica gel (Sigma-Aldrich). Silica was replaced with fresh desiccant as required (based on discolouring of silica gel) and leaf samples were allowed to completely desiccate prior to DNA extraction.

The utility of the SSR's developed in *C. miniata* and *C. nobilis* had not been assessed (Gao et al., 2012) in the entire genus and the initial point of departure was to determine the efficacy of these markers and their suitability to this phylogenetic and population genetic research. To this end, cross amplification of markers developed in *C. miniata* and *C. nobilis* (Gao et al., 2012) was undertaken for all six described *Clivia* species. Five samples per species was obtained from wild populations for this purpose. Primers for the entire panel of 10 polymorphic microsatellites (Gao et al., 2012) were ordered from Integrated DNA Technologies (USA). Forward primers of each microsatellite were labelled with a fluorescent dye at the 5’ end. Primer stock solutions were diluted according to manufactures instructions and kept at – 80°C, whilst working stocks were kept in the fridge at 4 °C to prevent freeze-thaw marker denaturation.

DNA extraction was undertaken using Zymo Quick-DNA™ Plant/Seed MiniPrep Kit. Beads from the ZR BashingBead™ Lysis Tube (Zymo Quick-DNA™ Plant/Seed MiniPrep Kit) were transferred to clean Eppendorf® Safe-Lock microcentrifuge tubes (ZR BashingBead™ Lysis Tubes did not fit in the disrupter). Leaf material was weighed to approximately 0.02 g and placed into the Eppendorf® tubes with Beads from the ZR BashingBead™ Lysis Tube (Zymo Quick-DNA™ Plant/Seed MiniPrep Kit). A TissueLyser II (Qiagen) was used for cellular disruption (30 s at maximum speed) prior to the addition of Bashingbead™ Buffer (Zymo Quick-DNA™ Plant/Seed MiniPrep Kit). Disrupted tissue from leaf samples was centrifuged (SCILOGEX D3024 High Speed Micro-Centrifuge) at 15 000 rpm for 30 s, after which BashingBead™ Buffer was added and the cellular disruption step was repeated. From here, steps as per the protocol laid out in the Zymo Quick-DNA™ Plant/Seed MiniPrep Kit were followed as this provided the best DNA yield and purity as assessed by a NanoDrop™2000 spectrophotometer (ThermoFisher Scientific). Extracted DNA was stored in the fridge at 4 °C when in use and kept at – 80°C when not in use. Approximately 300 extractions were carried out on leaf material from 10 different populations (four of *C. gardenii* and six of *C. robusta*) and additional extractions from *C. miniata*, *C. caulescens*, *C. nobilis* and *C. mirabilis* (5 samples each).

Initial PCR (polymerase chain reaction) optimization using the methods described by Gao et al. (2012) proved unsuccessful. A range of annealing temperatures (from 48 to 68 °C) and cycling conditions were used in combination with various Taq polymerases (TaKaRa Taq™, Takara Bio INC; KAPA2G™, Kapa Biosystems; KAPA3G™, Kapa Biosystems) during PCR (polymerase chain reaction) optimization. The best amplification of PCR product, as assessed from intensity of bands on a diagnostic agarose gel in relation to a KAPA™ Universal DNA Ladder (Kapa Biosystems), was obtained using KAPA3G ™ Taq DNA polymerase. Optimal PCR reactions (final reaction volume 25 µl) consisted of 1 µl of pure extracted template DNA, 12.5 µl KAPA buffer, 25 µM $MgCl_2$, 0.2 µl Taq DNA polymerase (KAPA3G™, Kapa Biosystems), 0.5 µl of each primer and 9.3 µl of deionised dH_2O. During optimization, cycling was varied as follows; initial denaturation of either 3, 4 or 5 min at 95 °C; denaturation from either 15, 20, 25 or 30s; annealing from 48 to 68 (2 °C intervals) at 15, 20, 25 or 30 s; extension from 30, 40, 50 and 60 s; final extension from 1, 3, 5, 8 and 10 min at 72 °C. Various combinations of the above-mentioned thermal profile, together with differing primer concentrations (from 0.1 to 0.5 µl) and differing DNA concentrations (pure; 1:10; 1:20; 1:50, 1:100) were used to obtain the best results. Optimal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 40s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final annealing step of 72 °C for 1 min. Over 1000 samples were generated of which a total of 832 samples were sent to the CAF (Central Analytical Facility, Stellenbosch University, South Africa) for sequencing. PCR product was screened for successful amplification using agarose gel electrophoresis for 35 minutes at 100 v. A 1.5 % agarose concentration was prepared, using 1.5 g agarose (Sigma-Aldrich) which was made up to a volume of 100 ml with TBE buffer, with the addition of 6 µl of ethidium bromide (Sigma-Aldrich). PCR products (3 µl) were mixed together with a loading dye (1 µl KAPA Loading dye) into wells in agarose gel to screen for successful product amplification. A molecular weight DNA ladder (3µl KAPA Express Ladder) was used to reference the fragment size of the bands. Successfully amplified PCR product was sent for fragment analysis to the CAF for sequencing. GeneMarker® v2.4.0 (Soft Genetics) was used to score genotyping.

Data was unobtainable for the following published loci (Gao et al., 2012); CM12, CM54, CM289, CN68 and CN106 despite numerous attempts to optimise cycling conditions (Table 1). It was initially hoped that a panel of all 10 makers would be available, but several hundred attempts to optimise markers involving hundreds of differing PCR cycles were not successful. Given the low success rate and inconsistent amplification of the SSR markers of (Gao et al., 2012), new SSR marker development was therefore undertaken in an attempt to obtain a panel of markers for species characterization and phylogeographical research of *C. gardenii* and *C. robusta*. Construction of an enriched DNA library and development of complementary oligonucleotides to those regions flanking microsatellite markers was developed by Ecogenics GmbH (Balgach, Switzerland). Here, next generation (Illumina) sequencing was applied via bioinformatics pipelines to identify microsatellites and an algorithm used to identify the most suitable primer regions that flank the markers. Leaf samples (0.05 g) from 10 individuals of *C. gardenii* (5 from one population and 5 from separate populations) were sent to Ecogenics, where an genomic library was constructed from size-selected DNA fragments ligated into SAULA/SAULB-linker (Armour et al., 1994) through enrichment by magnetic streptavidin bead selection with oligonucleotide and biotin-labelled repeats (Gautschi, Widmer, and Koella, 2000). The enriched library was analysed using GS FLX titanium reagents on a Roche 454 platform, providing 300 loci with flaking primer regions. From this, 31 potential markers were selected. A mix of di-, tri-, and tetranucleotide repeats were selected, with a sorting bias towards longer repeats. Loci were avoided where either forward of reverse primers would potentially anneal closer than 10 base-pairs to the repeat motif. Mononucleotide repeats longer than 6 base-pairs were also avoided in order to circumvent polymerase slipped strand mispairing (mononucleotide slippage). In order to facilitate downstream multiplexing, a mixture of amplicon sizes were selected.

Unlabelled primers (31 pairs) were ordered from Integrated DNA Technologies (USA) and diluted according to manufacturer’s instructions. PCR protocol was followed as above and PCR optimization followed similarly. The best amplification of PCR product was obtained using KAPA3G™ Taq DNA polymerase (Kapa Biosystems). Optimal PCR reactions (final reaction volume 12.5 µl) consisted of 0.5 µl of pure extracted template DNA, 6.25 µl KAPA buffer, 0.1 µl Taq DNA polymerase, 0.25 µl of each primer and 5.15 µl of deionised dH_2O. Optimal cycling conditions consisted of an initial denaturation at 95 ºC for 3 min, followed by 35 cycles at 95 ºC for 20s (denaturation), 64 ºC for 15 s (annealing), 72 ºC for 30 s (extension) and a final annealing step of 72 ºC for 1 min. PCR product was screened for successful amplification using agarose gel electrophoresis for 40 minutes at 75 v. A 3 % agarose gel was prepared, using 3 g agarose (Sigma-Aldrich) which was made up to a volume of 100 ml with TBE buffer, with the addition of 6 µl of ethidium bromide (Sigma-Aldrich).

Following optimization, a total of 55 samples, representing 11 SSR's (PCR product from 5 populations of *C. gardenii*) were sent to the CAF for analysis with a Agilent 2100 Bioanalyser using a dsDNA HS assay (Agilent Technologies, USA). Samples underwent a post-PCR purification before being analysed via an automated on-chip DNA electrophoresis system (Agilent Technologies, USA). Each lane (11 in total) of the chip consisted of a single locus where each of the 5 samples for a single marker was pooled. Results indicated that 5 of the 11 SSR's showed polymorphic signals (diploid codominant markers). Following this a 3K PerkinElmer LabChip® DNA X-Mark chip (PerkinElmer, USA) was used to screen 19 markers in a similar manner due to cost efficiency.

From these results 16 potential markers were selected and fluorescently labelled primers (labelled on the forward primer) were ordered from Integrated DNA Technologies (USA). Work on cross-amplification for all six described *Clivia* species began using the newly developed markers. The number of usable markers was reduced to only eight at this stage (Table 2). Unspecified annealing, low or no product amplification resulted in the discounting of the remainder of the markers and attempts to optimise proved unsuccessful. Over 500 samples were generated for marker optimization at this stage, of which 216 were sent for sequencing at the CAF. Successful cross amplification of the majority of markers was achieved for a number of the six described *Clivia* species (Table 3) and a primer note will be produced detailing this work but is beyond the scope of the current PhD.

Genotyping of *C. gardenii* and *C. robusta* entailed the generation of over 800 samples, 497 of which were sent for sequencing to the CAF. Genotyping proved troublesome as some primers worked inconsistently at genotyping. This may be due to differing DNA yields from the various samples as unspecified annealing made scoring difficult. Markers CG1, CG16 and CG23 achieved good amplification and to a lesser extent CG12. The remaining markers (Table 3) worked inconsistently and more work is required to produce a meaningful data set. This work will be continued at a later stage and will likely incorporate additional markers in order to achieve a complement of around 15 loci.

REFERENCES

ARMOUR, J. A., R. NEUMANN, S. GOBERT, AND J. A. JEFFREYS. 1994. Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics* 3: 599-605.

CONRAD, F., AND D. SNIJMAN. 2011. Systematics and Phylogeography of *Clivia*. *In* R. Fisher AND R. Dixon [eds.], *Clivia* Thirteen. *Clivia* Society, Pretoria, South Africa.

DIXON, R. 2005. *Clivia robusta* "maxima". *Clivia* 7: 62.

FELBERT, C. 2003. Map of known distribution of *Clivia* species. *Clivia* 5: 96-97.

GAO, W., H. ZHOU, Y. LIU, M.-R. LI, L.-F. NURMAMAT, Y.-Y. REN, AND H.-X. XIAO. 2012. Isolation and identification of fourteen microsatllite markers in *Clivia miniata* and *Clivia nobilis* (Amaryllidaceae). *International Journal of Molecular Sciences* 13: 9609-9614.

GAUTSCHI, B., A. WIDMER, AND J. KOELLA. 2000. Isolation and characterization of microsatellite loci in the dice snake (*Natrix tessellata*). *Molecular Ecology* 9: 2191-2193.

HUGHES, M., M. MÖLLER, D. U. BELLSTEDT, T. J. EDWARDS, AND M. WOODHEAD. 2004. EST and random genomic nuclear microsatellite markers for *Streptocarpus*. *Molecular Ecology Notes* 4: 36-38.

HUGHES, M., M. MÖLLER, D. U. BELLSTEDT, T. J. EDWARDS, AND M. DE VILLIERS. 2005. Refugia, dispersal and divergence in a forest archipelago: a study of *Streptocarpus in eastern South Africa*. *Molecular Ecology* 14: 4415-4426.

ROURKE, J. P. 2012. South African National Biodiversity Institute, Personal communication.

SPIES, P., J. P. GROBLER, AND J. J. SPIES. 2011. A review of phylogenetic relationships in the genus *Clivia*. *Philosophical Transactions in Genetics* 1: 168-207.

TABLES

Table 1: Number of genotypes obtained from published microsatellite markers (Gao et al., 2012) for each described *Clivia* species.

Clivia species	Number of individuals successfully sequenced for each locus (Gao et al., 2012)									
	CM9	CM12	CM54	CM65	CM103	CM137	CM289	CM357	CN68	CN106
C. robusta	5	0	0	5	5	0	0	0	0	0
C. gardenii	5	0	4	4	5	3	0	0	0	0
C. miniata	5	0	4	5	5	3	0	3	5	0
C. caulescens	5	0	0	5	5	0	0	1	0	0
C. nobilis	5	0	0	5	5	0	0	0	0	0
C. mirabilis	5	0	0	2	5	0	0	0	0	1

Table 2: Details of unpublished microsatellite markers

Locus	Repeat motif	Repeat length	Amplicon size	Dye	Primer sequences (5’-3’) Forward	Reverse
CG1	(AT)	19	138	HEX	TGTCAAACCATGAGCAACGC	AGGGACATTGCACAAAGCAG
CG12	(AAT)	9	234	FAM	TCGCGTGAAATGAGAGCAAC	GCTTGGGACAAATGGGGAAG
CG13	(ATA)	9	233	TET	ATGCTGACGGGGATGTAAGG	TCGTGCTACGACTCCTAAAG
CG15	(AAG)	8	196	TET	ATTGGGTTTGGATTCATCTTCC	GCAATGCAATTTTCTTTTCGTTTC
CG16	(AAT)	9	130	FAM	GGGGTAAGAATGTAAGGACTCG	AGCTCCTAATGAAACTAAACTAACC
CG23	(ATT)	12	131	HEX	GCAAGGGATGTCGTGAACAG	TCGAGTTCCTACGTTCCAGC
CG28	(ATAC)	9	245	FAM	CGTGTAACCCTTAGCAGAGC	TGCTTGCCTGATCTTGTTGC
CG30	(GATG)	10	194	FAM	ATCCTTTGCACCCCCGTTC	GGGCCATGTAGGGTATATGAGG

Table 3: Number of genotypes obtained from unpublished microsatellite markers for each described *Clivia* species.

	Number of individuals successfully sequenced for each locus							
Clivia species	CG1	CG12	CG13	CG15	CG16	CG23	CG28	CG30
C. robusta	5	5	5	0	5	5	5	5
C. gardenii	5	5	5	0	5	5	5	5
C. miniata	5	5	1	4	5	5	5	0
C. caulescens	5	5	5	0	5	5	5	3
C. nobilis	5	5	5	4	5	5	5	3
C. mirabilis	5	4	5	0	5	5	5	0

ROUND THE BEND: FUNCTIONAL CONSEQUENCES OF FLOWER CURVATURE, ORIENTATION AND PERCH POSITION FOR NECTAR FEEDING BY SUNBIRDS

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All authors contributed to the design of the study. Ian Kiepiel set up the experiments and recorded bird behaviour. Steven D. Johnson analysed the data. Steven D. Johnson wrote the paper with inputs from Ian Kiepiel and Alastair Robertson.

Mutualisms between nectarivorous birds and the plants they pollinate are functionally diverse. One striking contrast is between the virtually straight bills of nectarivorous birds that hover while feeding (the majority of hummingbirds) versus the decurved bills of those that perch while feeding (some hummingbirds and almost all passerine nectarivores). This is generally mirrored in the shapes of flowers pollinated by these avian functional groups. We hypothesized that perching nectarivorous birds such as sunbirds possess decurved bills because they probe in an arc motion and predicted that flowers with shape and orientation that enable birds to probe them in an arc will be preferred and also handled more quickly. To test this, we examined the responses of sunbirds to model flowers differing in curvature (straight or curved), orientation (facing upwards or downwards) and availability of a top perch (present or absent). Preferred perching position was strongly influenced by model shape; birds preferred to perch below curved models that face downwards and also handled these models most quickly in terms of time from landing to bill insertion. When both bottom and top perches were available, birds preferred the upper perch for all models, particularly those curved upwards. These results are consistent with the hypothesis that the curvature of flowers in perching bird mutualisms and the orientation of these flowers relative to the perch position are explained by preferences for flowers that can be handled most efficiently.

Keywords: bird pollination, floral syndromes, flower shape, nectar, optimal foraging, ornithophily, Nectariniidae, nectarivory, nectarivorous birds, passerine pollination, perching birds

In reality, the possession of a bill shaped in a particular way does not restrict that species to similarly-shaped flowers, and vice versa (Paton & Collins, 1989)

INTRODUCTION

It is well known that combinations of floral traits such as colour, rewards, and floral tube length influence which animals are attracted to flowers and the extent to which they effect pollen transfer. Flower shape and orientation are traits that can potentially play key roles in pollination mutualisms (Fenster, Armbruster, and Dudash, 2009; Campbell, Jurgens, and Johnson, 2016), including in bird pollination systems (Gill and Wolf, 1978; Montgomerie, 1984; Maglianesi et al., 2014; Ngcamphalala, Bailey, and Nicolson, 2018; Pauw, 2019; Sonne et al., 2019). Here we consider the functional consequences of flower shape, flower orientation and perch availability for nectar feeding by sunbirds. Although formal tests are rare (Lagomarsino et al., 2017; Sonne et al., 2019), it has been suggested that floral shape in bird–pollinated flowers corresponds to bill shape, with plants pollinated by straight-billed birds being more likely to have straight-tubed flowers, and those pollinated by birds with curved beaks being more likely to have curved-tube flowers (Stiles, 1975; Stein, 1992). Most hummingbirds hover while feeding and, on average, have straighter bills than do perching birds such as sunbirds and honeyeaters which have decurved bills (Paton and Collins, 1989). An interesting exception are sickle-billed hummingbirds that perch while feeding and have very strongly decurved bills (Stein, 1992; Lagomarsino et al., 2017). Remarkably, there are cases where female hummingbirds have curved beaks and mainly perch while feeding, while males of the same species have straighter beaks and mainly hover while feeding (Temeles et al., 2009). In honeycreepers which feed from a perching position, bill curvature is positively correlated with degree of nectarivory (Carothers, 1982).

Flower curvature is a hallmark of plant adaptation for pollination by sunbirds and other perching birds (Goldblatt, Manning, and Bernhardt, 1999; Goldblatt and Manning, 2006; Cronk and Ojeda, 2008). Curved flowers are also characteristic of many plants that are adapted for pollination by hermit hummingbirds that sometimes perch while feeding and have strongly decurved bills (Temeles et al., 2009). In their studies of East African sunbirds, Gill and Wolf (1978) found that species with relatively curved bills could extract nectar from curved flowers more quickly than species with straighter bills, a trend that was reversed when the same species fed on straight-tubed flowers. This suggests that handling efficiency could affect flower selection by sunbirds, but because the study by Gill and Wolf (1978) was limited to comparisons among two plant species which may vary in other characteristics besides flower shape, it has been difficult to reach general conclusions about the influence of flower shape on functional aspects of nectar feeding by sunbirds.

We hypothesized that the function of the curvature of the bills of most perching birds is that such bills follow an arc-like motion when they probe from a fixed perching position. A mechanical analogy would be the curved head of a pick or geological hammer. Indeed, curved bills are a feature of many birds, not just nectarivorous ones, that feed by probing from a standing position e.g. kiwi, ibises, and many sandpipers (Ferns and Siman, 1994; Nebel, Jackson, and Elner, 2005; Cunningham et al., 2010). Paton and Collins (1989) speculated that the curved bills of perching nectarivorous birds enable the tip to be inserted into a flower with "less stretching on the bird's part", which is essentially making the same point as ours about the arc-like feeding motion, although they make the alternative suggestion that curved bills may act as a hook, allowing birds to draw the entrance of flowers with flexible pedicels closer to the bird.

Paton and Collins (1989) suggested that selection on flowers pollinated by birds with curved bills would have favoured those that "curved back towards the usual perching sites". However, they also point out that in many instances, birds with curved bills can probe straight flowers, especially if the corolla tube is short or broad and the pedicel is flexible such that the flower can be re-orientated into the feeding arc. Other authors have suggested that the function of curved corolla tubes is not that they are preferred by birds with curved beaks, but rather that the curved corolla tubes exclude other floral visitors (Stein, 1992). The orientation of bird-pollinated flowers has also been debated, with some authors suggesting that the high frequency of pendant orientation in hummingbird-pollinated flowers is related to protection of nectar from rain (Aizen, 2003). Pendant orientation is also common in flowers pollinated by perching birds (Goldblatt, Manning, and Bernhardt, 1999), but this may be more strongly related to the use of the stem below the flowers as a perch. Experimental studies of the consequences for nectar feeding by perching birds when flowers vary in tube curvature and orientation of the tube relative to perch positions are needed to resolve some of these questions.

We predicted that sunbirds would feed more efficiently from curved flowers, particularly when the flowers are curved inwards towards a perch. To test this prediction, we designed model "inflorescences" with flowers that varied in curvature, flower orientation and perch availability. Specifically, we asked whether (1) birds would prefer to land on "inflorescences" with flowers curved towards the perch position (2) whether birds would prefer to feed from a perching position that enabled an arc-like probe into a curved flowers, (3) whether birds would probe flowers that curve towards the perch more than those that did not do so, (4) whether birds would consume more nectar from flowers that curved towards the perch and (5) whether birds would handle flowers that curved towards the perch more quickly than those that did not.

METHODS

Sunbird feeding trials

BIRD HOUSING AND DIET - Five male Amethyst Sunbirds *(Chalcomitra amethystina*, mean ± SE body mass = 14.66 ± 0.14 g; mean ± SE, culmen lengths 31.14 ± 0.28 mm) were captured using mist nets in

Pietermaritzburg, South Africa in November 2014. They were housed at the Animal House of the University of KwaZulu-Natal, Pietermaritzburg, in 300 x 450 x 900 mm cages in a 12:12 light: dark photoperiod at a temperature of 25 ± 1°C. Birds were fed a maintenance diet which was refreshed twice daily to eliminate protein separation and nutrient spoiling (08h00 and 15h00). Water and fruit flies were supplied *ad libitum*, cages cleaned daily, and feeders sterilised prior to every feeding (80°C dishwasher cycle). Maintenance diet consisted of a 20% sterilised sucrose solution, which was supplemented with 5 ml of Avi-Sup Soluble Vitamins (AviProducts, Durban, RSA) and 15 ml of Ensure Nutrition Powder (Abbott Laboratories, Hoofddorp, The Netherlands) per litre of liquid feed.

Models and experimental layout – Artificial flowers representing differing floral curvature (straight or curved) and orientation (facing upwards or downwards) were constructed from 12 mm diameter transparent silicone tubing. Four distinct models were constructed, representative of floral morphologies encountered by specialized passerine nectarivores or hummingbirds (see Figure 1 for models). Models were constructed using a single piece of silicone tubing 16 cm in length, which was folded and tightly crimped in the middle with a white cable tie to create two discrete air-tight ‘floral tubes’ (i.e. flowers) that were 6 cm in length, which could be filled with artificial nectar solution. The air-tight construction of the tube ensured that nectar was held in the tube even if ‘flowers’ faced downwards. We marked a distance 31 mm from the entrance and used an additional wire crimp to narrow the tubes at this point. This distance matched the length of the culmen and meant that the birds could only access this narrowed section with their tongues. This design simulates the morphology of bird-pollinated flowers, which usually have a broader section of the floral tube that accommodates the bill and a narrow base containing nectar (Goldblatt, Manning, and Bernhardt, 1999; Goldblatt and Manning, 2006). This additional crimp also helped to keep the nectar in place even when the flower faced downwards.

We attached two strands of 1 mm stainless steel wire on each side of the ‘floral tube’ (anteroposterior axis), held by three (per flower) equidistant white 2.5 mm cable ties, and, in the case of the models with curved tubes, we bent this wire to attain curvature. The entrance of these artificial flowers was wrapped with a thin layer of 12 mm red tape (3M Scotch® Brand Tape, RSA) to act as a visual aid for feeding. Each model had two ‘flowers’, one facing the left and the other to the right (Figure 1). Model flowers were attached to a 91.5 cm long unpainted wooden dowel (diameter 9 mm) using cable ties. These were held in place using retort stands so that the artificial flowers came to a height of approximately 1 m. For convenience, models with different combinations of tube curvature and orientation are referred to as *Clivia*-type (Figure 1a), *Salvia*-type (Figure 1b), *Babiana*-type (Figure 1c), and *Penstemon*-type (Figure 1d) on the basis of their similarity to bird-pollinated flowers in these genera (Wilson et al., 2004; Anderson, Cole, and Barrett, 2005; Benitez-Vieyra et al., 2014; Kiepiel and Johnson, 2014). Tubes of *Clivia*-type models were curved downwards and orientated at 90° to the perch axis (Figure 1a). Tubes of *Salvia*-type models were curved downwards but orientated upwards at a 135° angle relative to the perch axis (Figure 1b & f). Tubes of *Babiana*-type models were curved upwards and orientated at a 135° angle relative to the perch axis (Figure 1c). Tubes of *Penstemon*-type models were straight and orientated at 90° to the perch axis (Figure 1d). We created models where the flowers were placed at the top of the dowel rod (Figure 1a-d), thus providing a bottom perch, and those placed 20 cm from the top (Figure 1e-h), thus providing an additional top perch. To avoid excessive numbers of choices, we conducted separate trials for models without top perches and those with top perches.

We conducted 23 trials involving models without top perches over a period of 12 days, followed by 13 trials involving models with top perches over a period of seven days. For each feeding trial a single bird was moved from the Animal House into an outdoor flight cage (1 × 2 × 3 m). Birds were given half an hour to settle down prior to the commencement of each trial. We filled the narrowed section of the tube of each model flower up to the 31 mm mark with 1.5 ml of 20% sucrose solution and placed the crimp at this point. Trials were undertaken during the morning from 08h00 to 12h00. Models were spaced equidistantly with dowel rods 300 mm apart at the rear of a flight cage with positions randomised prior to each three-hour experiment. Automated motion-activated cameras (Bushnell® 14 Mp Natureview Cam HD) were placed inside the cage 1.5 m from the models in order to attempt to capture feeding behaviour. Camera traps were not, however, always reliably triggered by the birds, with the consequence that observations and notes were made concurrently. We recorded each model that a bird landed upon, whether the bird fed, which ‘flower’ on each model was fed upon (i.e. left or right), the probe duration and the handling time (from landing to bill insertion). In models with top perches, we recorded whether the birds used the bottom or top perch or perched on the flower itself. After three hours, the crimp was removed from the 31 mm mark on the tube and, with the tube angled upwards, residual nectar was measured using callipers in the ‘floral’ tube (i.e. distance from original mark to residual nectar) and the bird returned to the Animal House. The distance between the original mark and the final nectar level was converted to volume using the equation for the volume of a cylinder. After each trial models were immediately sterilized using alcohol and flushed with plenty of water to clean them for the next trial.

STATISTICAL ANALYSIS - Data were analysed in SPSS 26 (IBM Corp) using generalized linear mixed effects models (GLMMs) that accounted for the distribution of the data and design of the study. We performed separate analyses of experiments in which models lacked a top perch and those in which models had a top perch because these experiments were done separately. Analyses of the number of landings and number of probes incorporated a negative binomial distribution and log link function. Analyses of the proportion of birds that fed from above the model incorporated a binomial distribution (“events/trials” structure) and logit link function. Analyses of the amount of nectar consumed and the handing time incorporated a Gaussian distribution and identity link function. In the analyses of bird landings we included the natural log of the time in hours for each time block as an offset in order to obtain a rate of landings per hour. Analyses of handling time were restricted to birds that perched on the dowel rod, as perching on the model flowers was considered biologically unrealistic as the corolla of most natural flowers cannot support the weight of a bird. To account for non-independence among samples, we treated trial nested in bird identity as a random effect. Bird identity was not included as a separate random effect as the number of levels (5) was too low to reliably estimate the covariance matrix. We used the Kenward-Rogers procedure to estimate denominator degrees of freedom for F statistics. Multiple comparisons among means were based on the sequential Šidák method. For graphical representation of mean proportions and standard errors, data were back-transformed from the logit or log scales.

RESULTS

We recorded 676 landings (mean of 29.4 per trial) on model inflorescences that lacked a top perch and 413 landings (mean of 31.7 per trial) on model inflorescences that had a top perch. The mean overall number of landings per hour did not vary among model types in experiments in which models lacked a top perch ($F_{3, 177}$ = 0.157, P = 0.92; Figure 2a) and in those where a top perch was present ($F_{3,95}$ = 0.201, P = 0.89; Figure 2b). The number of landings per hour differed between time blocks in the trials with models lacked a top perch ($F_{1, 177}$ = 5.90, P = 0.016; Figure 2a), but not when a top perch was present ($F_{3,95}$ = 0.83, P = 0.36; Figure 2b). There were no overall significant interactions between model type and time block on landings, either for models without a top perch ($F_{3,177}$ = 1.73, P = 0.16) or for those with a top perch ($F_{3,95}$ = 0.42, P = 0.73). There was, however, a marked decrease between the first and second time block in the rate of landings on the *Clivia*-type model lacking a top perch ($F_{1,177}$ = 6,88, P = 0.009, Figure 2a), while contrasts between the two time blocks were not significant for landings on any of the other model types.

The feeding positions adopted by birds varied significantly among models lacking a top perch ($F_{3,83}$ = 29.07, P < 0.0001; Figure 2c & d). Birds fed almost exclusively from beneath the *Clivia*-type and *Penstemon*-type models and mostly from below the *Salvia*-type model, but almost always chose to feed from above the *Babiana*-type models (Figure 2c). Feeding positions also varied significantly among models when a top perch was present ($F_{3,41}$ = 16.37, P < 0.0001). The top perch was strongly preferred for feeding on all models in these experiments, apart from the *Clivia*-type model where 50% of birds chose to feed from the lower perch. (Figure 2d).

The overall number of probes received did not vary significantly among model types lacking a top perch ($F_{3,66}$= 1.41, P =0.245), nor among those with top perches ($F_{3,48}$= 1.66, P = 0.187; Figure 2e-f). Nectar volume consumed by birds differed significantly among models lacking top perches ($F_{3,66}$ = 3.28, P = 0.026), but not among those with top perches ($F_{3,44}$ = 0.059, P = 0.98). The amount of nectar consumed from the *Penstemon*-type model was significantly lower than that consumed from the other model types when no top perch was present (Figure 2g).

Handling time by birds differed significantly among models when no top perch was present ($F_{3,161}$ = 8.03, P < 0.001; Figure 3a). The *Clivia*-type model was handled most efficiently by sunbirds when no top perch was present and birds perched below the flower (Figure 3a). There was no overall difference in bird handling time among models with top perches ($F_{3,91}$ = 0.58, P = 0.63), but there was a strong interaction between model type and perch position for handling time with models with top perches ($F_{3,94}$ = 7.59, P < 0.001). The *Clivia*-type models with a top perch were handled most efficiently when birds perched below the model (Figure 3b). When birds used the top perch, they handled the *Clivia*-type model least efficiently (Figure 3b).

DISCUSSION

The results of this study are consistent with our initial hypothesis that floral curvature and orientation in plants pollinated by perching birds function to accommodate the arc-like motion of feeding from flowers. Flower curvature, flower orientation, and perch availability strongly influenced the feeding position assumed by birds (Figure 1, Figure 2 c & d) and flower handling time (Figure 3).

The overall preference of sunbirds for feeding from above flowers, particularly when a top perch is present (Figure 2, Figure 3 c & d) is seemingly at odds with the architecture of most sunbird-pollinated plants which provide a perch in the form of a sturdy inflorescence stem below the flowers. Our interpretation is that perch position in most plants may simply be a spandrel (Gould and Lewontin, 1979), i.e. a design constraint, in this case based on the development of inflorescences from a flowering stem that grows upwards, such that flowers are invariably positioned above the stem. An interesting exception is *Babiana ringens* Ker Gawl., a South African plant that provides a perch above the flowers in the form of a sterile inflorescence stem (Anderson, Cole, and Barrett, 2005). The flowers of *B. ringens* are curved and orientated upwards (Figure 4d). In our study, birds preferred to feed on *Babiana*-type models from above, especially when a perch was provided above the flowers. The evolution of perches is not a factor that is often considered in the design of inflorescences (Westerkamp, 1990), but evidence suggests that shifts to bird pollination in plants are often accompanied by the evolution of perches (Figure 4) which can be in the form of thicker reinforced stems (Siegfried, Rebelo, and Prys-Jones, 1985), modified petals as in *Strelitzia* (Frost and Frost, 1981), sterile inflorescences as in *Babiana* (Anderson, Cole, and Barrett, 2005; Figure 4d) or, in the case of plants that transfer pollen via bird feet, even a particularly narrow inflorescence with flowers that are clasped by bird feet (Johnson and Brown, 2004). In a few very rare cases, plants adapted for pollination by sunbirds lack perches altogether and have geoflorous flowers pollinated by birds that perch on the ground (Hobbhahn and Johnson, 2015), or have weakly developed perches and are pollinated by sunbirds that frequently hover (Padyšáková and Janeček, 2016). A key evolutionary consequence of perching behaviour by birds is often the clustering of flowers into dense aggregations that can be probed from a single perching position (Cronk and Ojeda, 2008; Ford and Johnson, 2008). Indeed, the densely clustered upward-facing flowers in inflorescences of bird-pollinated species in the Proteaceae also frequently serve as a top perch for foraging sunbirds (Collins and Rebelo, 1987; Figure 4f).

Total nectar consumption was not strongly affected by model shape in our experiments. The highest levels of nectar consumption were recorded for the *Clivia*-type models and the lowest for *Penstemon*-type models when top perches were absent (Figure 2g). Nectar consumption was similar for all model types when top perches were available (Figure 2h). This could be explained by the birds' being able to exploit all model types equally, given the availability of perches above and below the model flowers. Flower orientation, similarly, did not affect nectar consumption in a study based on a smaller sunbird species (Ngcamphalala, Bailey, and Nicolson, 2018). However, their experiment involved the placement of model flowers on a mesh feeding board such the birds could perch in any position.

In our experiments involving *C. amethystina* sunbirds, each individual bird consumed about 0.5 ml of the 1.5 ml of 20% sugar solution we placed in each model or around 4 ml in total during the three-hour trial period. Similar rates of *ad libitum* consumption have been reported for malachite sunbirds, *Nectarinia famosa*, which have approximately the same body mass as *C. amethystina* (Brown, Downs, and Johnson, 2010) as well as for double collared sunbirds *Cinnyris chalybea* (Lotz and Nicolson, 1999) and cape sugarbirds, *Promerops cafer* (Jackson, Nicolson, and Lotz, 1998), suggesting that our provisioning of sugar solution in the model flowers was biologically realistic. However, it is possible that the flowers that were most depleted early in the experiment would become less attractive later in the experiment as the sugar solution became harder to access. This may explain the significant contrast for visitation between time blocks for the *Clivia*-type flowers lacking top perches, even though the overall interaction between model and time block was not significant (Figure 2a). Amethyst sunbirds can extend their tongues about 41 mm beyond the end of their bills (Robertson & Johnson, unpublished data), yet the maximum drop in the level of the sugar solution was only 24.6 mm for the models without top perches and 30 mm for the models with top perches, suggesting that no model flower was depleted of sugar solution to a level at which it was functionally non-rewarding, but it is well known that birds do not feed as effectively when their tongues are fully stretched out (Montgomerie, 1984; Paton and Collins, 1989). The birds in our study fed for short periods (c. 5 s per model flower) throughout the experiment, despite the essentially unlimited nectar in each model, suggesting that they had little ability to store large amounts of nectar. This need for frequent small meals of nectar is consistent with anatomical studies which suggest that sunbirds lack a well-developed crop (Mbatha, Downs, and Penning, 2002).

In our study, inflorescence architecture and flower curvature did not affect the probing time by sunbirds. Castellanos et al. (2004) found that handling time (total visit duration including probing time) by hummingbirds increased when *Penstemon* flowers were manipulated into a pendant position. However, Montgomerie (1984) found no effect of corolla angle (between 0 and 135 degrees) of model flowers on probing time by hummingbirds. Collins (2008), also using model flowers, found that flower curvature had no effect on probing time by hummingbirds, but increased probing time by honeyeaters.

The strongest effect of inflorescence architecture in our study was on handling time, measured as the time from perching to the start of feeding (Figure 3). Birds that were able to perch below flowers that curved downwards (i.e. the *Clivia*-type models) handled these models most efficiently (Figure 3). For *Clivia*-type models with a top perch, birds took almost six seconds before feeding when landing on the upper perch and only one second when landing on the lowers perch (Figure 3b). This pattern was reversed, but the difference was less marked, when flowers curved upwards (Figure 3b). From video footage (Video S1) of our experiments, it is evident that the birds handled the *Clivia*-type models effortlessly when they used the lower perch. It is most likely that these models fitted the natural arc motion of sunbird probing from a perch, but we cannot exclude the possibility that birds acquired experience of how to handle the *Clivia*-type models before they were captured, as this model approximates the most common architecture of sunbird-pollinated flowers.

We caution against the idea that handling time and the general efficiency of feeding behaviour is the only selection pressure on floral design. Many if not the majority of hummingbird-pollinated flowers are pendant, but metabolic studies show that it is actually more energetically costly for hummingbirds to feed from pendant flowers than from horizontal flowers (Sapir and Dudley, 2013). Aizen (2003) suggested that protection of nectar from dilution by rain could be the primary selective factor that accounts for the evolution of pendant orientation in hummingbird-pollinated flowers. However, in the case of sunbird-pollinated plants, we think that the main factor that accounts for pendant floral orientation is that this allows flowers to face the perching position, which is usually below the flowers in the case of upright inflorescences (Figure 4a). Pendant orientation characterizes the flowers of many sunbird-pollinated aloes (Botes, Johnson, and Cowling, 2008) which occur in arid regions, suggesting that rain is unlikely to be a factor in selection on flower orientation in those species. There is also evidence that pendant flowers are less likely to be visited by certain insects (Fulton and Hodges, 1999)

Although some sunbird species will readily hover to feed (Geerts and Pauw, 2009; Janecek et al., 2011; Wester, 2013; Padyšáková and Janeček, 2016), instances of hover-feeding by the amethyst sunbirds in our study were extremely rare. We recorded only three cases of hovering among the 521 visits to models that lacked a top perch (Video S1) and no cases of hovering among the 293 visits to models with a top perch. Hovering behaviour has also not been reported in the studies of flower visitation by amethyst sunbirds studied in the field (Botes, Johnson, and Cowling, 2008; Hargreaves, Harder, and Johnson, 2010). However, it is also possible that sunbirds in our study did not hover because all of the flowers could be accessed from perches or by perching on the tubular part of the models. Cameroon sunbirds (*Cyanomitra oritis*) are more likely to hover feed from flowers of *Impatiens sakeriana* Hook.f. when the entrance is further away from a perch, but sometimes hover even when a perching position is available (Padyšáková and Janeček, 2016).

Although several studies have identified differences in the behaviour of birds feeding on plant species that differ in floral morphology, such studies do not control for other variables besides morphology, making it difficult to reach firm conclusions about the functional consequences of floral morphology. Model flowers allowed us to control for variables such as colour, size, height, and nectar volume, and to vary only key characters of interest (Smith et al., 1996; Newman, Anderson, and Johnson, 2012). This is a general advantage of the use of model flowers for studies of plant-pollinator interactions (Policha et al., 2016), but of course model flowers also have disadvantages. The chief disadvantage of the model flowers used in this experiments was that they were rigid enough to allow birds to occasionally use them as a perch (Figure 1), although we were able to exclude such "unnatural" behaviour in certain of our analyses such as the analysis of handling times, but we were unable to exclude the effects of such behaviour on other variables such as total nectar consumption.

In conclusion, this study identifies strong effects of inflorescence architecture and flower shape on aspects of sunbird feeding behaviour, notably perching position and overall handling time. These findings provide a basis for selection on floral traits, but confirmation of sunbird-mediated selection for flowers that are curved towards a perch will require experimental manipulation of real flowers (Anderson, Cole, and Barrett, 2005; Campbell, 2009; Liu et al., 2013; Hargreaves, Langston, and Johnson, 2019) or studies of phenotypic selection on plants that show natural variation in these traits.

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REFERENCES

AIZEN, M. A. 2003. Down-facing flowers, hummingbirds and rain. *Taxon* 52: 675-680.

ANDERSON, B., W. W. COLE, AND S. C. H. BARRETT. 2005. Specialized bird perch aids cross-pollination. *Nature* 435: 41-42.

BENITEZ-VIEYRA, S., J. FORNONI, J. PEREZ-ALQUICIRA, K. BOEGE, AND C. A. DOMINGUEZ. 2014. The evolution of signal-reward correlations in bee- and hummingbird-pollinated species of *Salvia*. *Proceedings of the Royal Society B-Biological Sciences* 281.

BOTES, C., S. D. JOHNSON, AND R. A. COWLING. 2008. Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering phenology. *Oikos* 117: 875-882.

BROWN, M., C. T. DOWNS, AND S. D. JOHNSON. 2010. Concentration-dependent sugar preferences of the malachite sunbird (*Nectarinia famosa*). *Auk* 127: 151-155.

CAMPBELL, D. R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103: 1557-1566.

CAMPBELL, D. R., A. JURGENS, AND S. D. JOHNSON. 2016. Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices. *New Phytologist* 210: 333-342.

CAROTHERS, J. H. 1982. Effects of trophic morphology and behavior on foraging rates of three Hawaiian honeycreepers. *Oecologia* 55: 157-159.

CASTELLANOS, M. C., P. WILSON, AND J. D. THOMSON. 2004. 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* 17: 876-885.

COLLINS, B. G. 2008. Nectar intake and foraging efficiency: Responses of honeyeaters and hummingbirds to variations in floral environments. *Auk* 125: 574-587.

COLLINS, B. G., AND T. REBELO. 1987. Pollination biology of the Proteaceae in Australia and southern Africa. *Australian Journal of Ecology* 12: 387-421.

CRONK, Q., AND I. OJEDA. 2008. Bird-pollinated flowers in an evolutionary and molecular context. *Journal of Experimental Botany* 59: 715-727.

CUNNINGHAM, S. J., M. R. ALLEY, I. CASTRO, M. A. POTTER, M. CUNNINGHAM, AND M. J. PYNE. 2010. Bill morphology of ibises suggests a remote-tactile sensory system for prey detection. *Auk* 127: 308-316.

FENSTER, C. B., W. S. ARMBRUSTER, AND M. R. DUDASH. 2009. Specialization of flowers: is floral orientation an overlooked first step? *New Phytologist* 183: 502-506.

FERNS, P. N., AND H. Y. SIMAN. 1994. Utility of the curved bill of the curlew *Numenius arquata* as a foraging tool. *Bird Study* 41: 102-109.

FORD, C. M., AND S. D. JOHNSON. 2008. Floral traits, pollinators and breeding systems in *Syncolostemon* (Lamiaceae). *Plant Systematics and Evolution* 275: 257-264.

FROST, S. K., AND P. G. H. FROST. 1981. Sunbird pollination of *Strelitzia nicolai*. *Oecologia* 49: 379-384.

FULTON, M., AND S. A. HODGES. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society of London, Series B, Biological Science* 266: 2247-2252.

GEERTS, S., AND A. PAUW. 2009. African sunbirds hover to pollinate an invasive hummingbird-pollinated plant. *Oikos* 118: 573-579.

GILL, F. B., AND L. L. WOLF. 1978. Comparative foraging efficiencies of some montane sunbirds in Kenya. *Condor* 80: 391-400.

GOLDBLATT, P., AND J. C. MANNING. 2006. Radiation of pollination systems in the iridaceae of sub-Saharan Africa. *Annals of Botany* 97: 317-344.

GOLDBLATT, P., J. C. MANNING, AND P. BERNHARDT. 1999. Evidence of bird pollination in Iridaceae of southern Africa. *Adansonia (Paris)* 21: 25-40.

GOULD, S. J., AND R. C. LEWONTIN. 1979. The spandrels of San-Marco and the Panglossian paradigm - a critique of the adaptationist program. *Proceedings of the Royal Society of London Series B-Biological Sciences* 205: 581-598.

HARGREAVES, A. L., L. D. HARDER, AND S. D. JOHNSON. 2010. Native pollen thieves reduce the reproductive success of a hermaphroditic plant, *Aloe maculata*. *Ecology* 91: 1693-1703.

HARGREAVES, A. L., G. T. LANGSTON, AND S. D. JOHNSON. 2019. Narrow entrance of short-tubed Aloe flowers facilitates pollen transfer on long sunbird bills. *South African Journal of Botany* 124: 23-28.

HOBBHAHN, N., AND S. D. JOHNSON. 2015. Sunbird pollination of the dioecious root parasite *Cytinus sanguineus* (Cytinaceae). *South African Journal of Botany* 99: 138-143.

JACKSON, S., S. W. NICOLSON, AND C. N. LOTZ. 1998. Sugar preferences and "side bias" in Cape sugarbirds and lesser double-collared sunbirds. *Auk* 115: 156-165.

JANECEK, S., E. PATACOVA, M. BARTOS, E. PADYSAKOVA, L. SPITZER, AND R. TROPEK. 2011. Hovering sunbirds in the Old World: occasional behaviour or evolutionary trend? *Oikos* 120: 178-183.

JOHNSON, S. D., AND M. BROWN. 2004. Transfer of pollinaria on bird's feet: a new pollination system in orchids. *Plant Systematics and Evolution* 244: 181-188.

KIEPIEL, I., AND S. D. JOHNSON. 2014. Shift from bird to butterfly pollination in *Clivia* (Amaryllidaceae). *American Journal of Botany* 101: 190-200.

LAGOMARSINO, L. P., E. J. FORRESTEL, N. MUCHHALA, AND C. C. DAVIS. 2017. Repeated evolution of vertebrate pollination syndromes in a recently diverged Andean plant clade. *Evolution* 71: 1970-1985.

LIU, Z.-J., L.-J. CHEN, K.-W. LIU, L.-Q. LI, W.-H. RAO, Y.-T. ZHANG, G.-D. TANG, AND L.-Q. HUANG. 2013. Adding perches for cross-pollination ensures the reproduction of a self-incompatible orchid. *Plos One* 8: e53695.

LOTZ, C. N., AND S. W. NICOLSON. 1999. Energy and water balance in the lesser double- colared sunbird (*Nectarina chalybea)* feeding on different nectar concentrations. *Journal of Comparative Physiology B (Biochemical, Systematic, and Environmental Physiology)* 169: 200-206.

MAGLIANESI, A. M., N. BLUETHGEN, K. BOEHNING-GAESE, AND M. SCHLEUNING. 2014. Morphological traits determine specialization and resource use in plant-hummingbird networks in the neotropics. *Ecology* 95: 3325-3334.

MBATHA, K., C. T. DOWNS, AND M. PENNING. 2002. Nectar passage and gut morphology in the Malachite Sunbird and the Black-capped Lory: implications for feeding in nectarivores. *Ostrich* 73: 138-142.

MONTGOMERIE, ROBERT D. 1984. Nectar extraction by hummingbirds: response to different floral characters. *Oecologia* 63: 229-236.

NEBEL, S., D. L. JACKSON, AND R. W. ELNER. 2005. Functional association of bill morphology and foraging behaviour in calidrid sandpipers. *Animal Biology* 55: 235-243.

NEWMAN, E., B. ANDERSON, AND S. D. JOHNSON. 2012. Flower colour adaptation in a mimetic orchid. *Proceedings of the Royal Society B-Biological Sciences* 279: 2309-2313.

NGCAMPHALALA, C. A., I. E. BAILEY, AND S. W. NICOLSON. 2018. Nectar intake and foraging efficiency: the responses of sunbirds to flower morphology. *Journal of Ornithology* 159: 1031-1041.

PADYŠÁKOVÁ, E., AND Š. JANEČEK. 2016. Sunbird hovering behavior is determined by both the forager and resource plant. *Biotropica* 48: 687-693.

PATON, D. C., AND B. G. COLLINS. 1989. Bills and tongues of nectar-feeding birds - a review of morphology, function and performance, with intercontinental comparisons. *Australian Journal of Ecology* 14: 473-506.

PAUW, A. 2019. A bird's-eye view of pollination: Biotic interactions as drivers of adaptation and community change. *Annual Review of Ecology, Evolution and Systematics* 50: 477-502.

POLICHA, T., A. R. DAVIS, M. BARNADAS, B. M. DENTINGER, R. A. RAGUSO, AND B. ROY. 2016. Disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic 3D printed flowers. *New Phytologist* 210: 1058-1071.

SAPIR, N., AND R. DUDLEY. 2013. Implications of floral orientation for flight kinematics and metabolic expenditure of hover-feeding hummingbirds. *Functional Ecology* 27: 227-235.

SIEGFRIED, W. R., A. G. REBELO, AND R. P. PRYS-JONES. 1985. Stem thickness of *Erica* plants in relation to avian pollination. *Oikos* 45: 153-155.

SMITH, C. E., J. T. STEVENS, E. J. TEMELES, P. W. EWALD, R. J. HEBERT, AND R. L. BONKOVSKY. 1996. Effect of floral orifice width and shape on hummingbird-flower interactions. *Oecologia* 106: 482-492.

SONNE, J., T. B. ZANATA, A. M. M. GONZALEZ, N. L. C. TORRES, J. FJELDSA, R. K. COLWELL, B. A. TINOCO, et al. 2019. The distributions of morphologically specialized hummingbirds coincide with floral trait matching across an Andean elevational gradient. *Biotropica* 51: 205-218.

STEIN, B. A. 1992. Sicklebill hummingbirds, ants, and flowers. *BioScience* 42: 27-33.

STILES, F. G. 1975. Ecology, flowering phenology, and hummingbird pollination of some Costa Rican *Heliconia* species. *Ecology* 56: 285-301.

TEMELES, E. J., C. R. KOULOURIS, S. E. SANDER, AND W. J. KRESS. 2009. Effect of flower shape and size on foraging performance and trade-offs in a tropical hummingbird. *Ecology* 90: 1147-1161.

WESTER, P. 2013. Sunbirds hover at flowers of *Salvia* and *Lycium*. *Ostrich* 84: 27-32.

WESTERKAMP, C. 1990. Bird-flowers: hovering versus perching exploitation. *Botanica Acta* 103: 366-371.

WILSON, P., M. C. CASTELLANOS, J. N. HOGUE, J. D. THOMSON, AND W. S. ARMBRUSTER. 2004. A multivariate search for pollination syndromes among penstemons. *Oikos* 104: 345-361.

Figure Legends

Figure 1. The design of the model flowers and the typical feeding positions assumed by sunbirds on these models. (a) *Clivia*-type with no top perch. (b) *Salvia*-type with no top perch, (c) *Babiana*-type with no top perch. (d) *Penstemon*-type with no top perch. (e) *Clivia*-type with top perch. (f) *Salvia*-type with top perch. (g) *Babiana*-type with top perch. (h) *Penstemon*-type with top perch.

Figure 2. Responses of sunbirds to the architecture of model flowers without top perches (left panels) and with top perches (right panels). (a-b) Number of landings per hour for the first hour of the experiment versus the remaining two hours of the experiment. (c-d) The proportion of birds that fed from above the model. (e-f). Number of probes. (g-h) Nectar consumed from both flowers. Values are means ± SE. Means that share letters are not significantly different.

Figure 3. Handling time, taken as the time between landing and full bill insertion, for sunbirds on models of different architecture. Only cases where birds perched on the wooden dowel were considered - cases where birds used the model flower as a perch were discarded as unrealistic. (a) Models lacking a top perch. (b) Models with a top perch.

Figure 4. Use of perches by sunbirds feeding on flowers. (a) White-bellied sunbird (*Cinnyris talatala*) using a perching position below flowers of *Clivia gardenii* Hook. (Amaryllidaceae). (b) Greater double-collared sunbird *Cinnyris afer* feeding on flowers of *Crocosmia paniculata* (Klatt) Goldblatt (Iridaceae) The branching inflorescence stems offer a variety of perching positions below and above the flowers. (c). Amethyst sunbird *Chalcomitra amethystina* feeding on *Erythrina lysistemon* Hutch (Fabaceae). Flowers curve in towards the stem which is often used as an apical perch. (d) The sterile flowering stem of *Babiana ringens* (Iridaceae) provides an apical perch for birds feeing on the vertically orientated flowers. (e) Amethyst sunbird using the petal sheath and anthers as a perch while feeding from flowers of *Strelitzia reginae* Aiton (Strelitziaceae). (f). Malachite sunbird *Nectarinia famosa* using the stiff florets of *Protea caffra* Meisn. as a top perch position. Photos: SD Johnson. Scale bars 20 mm.

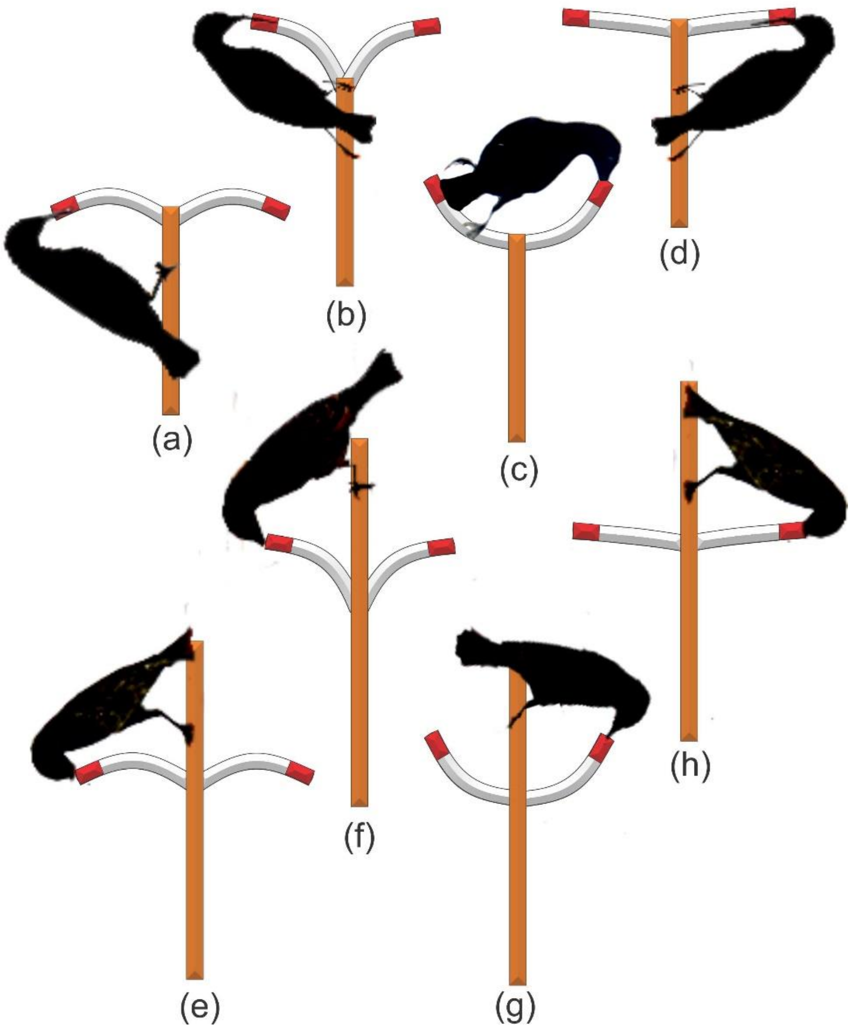

Figure 1.

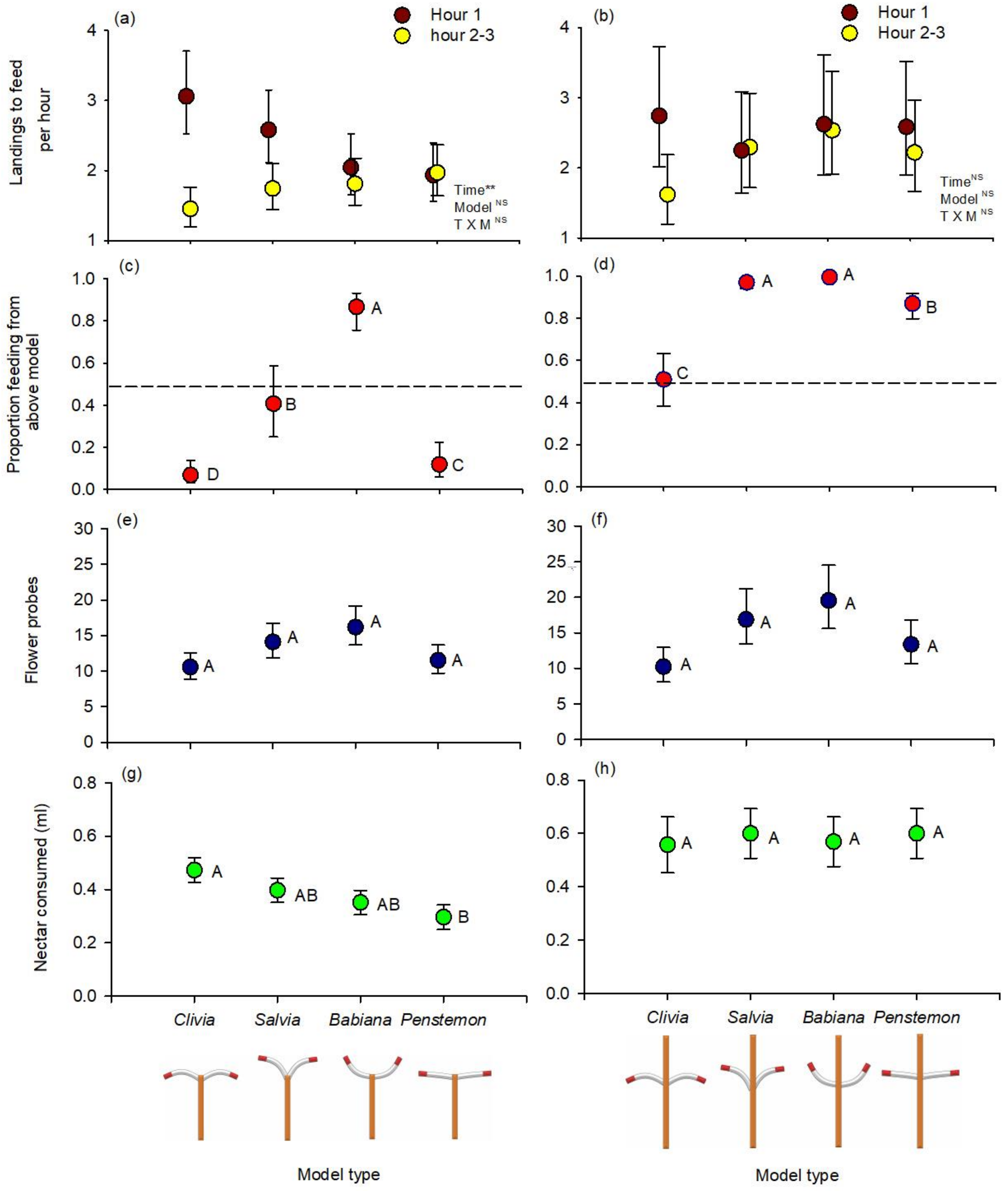
(a)
Hour 1
hour 2-3
Landings to feed per hour
1
2
3
4
Time**
Model NS
T X M NS
(b)
Hour 1
Hour 2-3
1
2
3
4
Time NS
Model NS
T X M NS
(c)
Proportion feeding from above model
0.0
0.2
0.4
0.6
0.8
1.0
D
B
A
C
(d)
0.0
0.2
0.4
0.6
0.8
1.0
C
A
A
B
(e)
Flower probes
0
5
10
15
20
25
30
A
A
A
A
(f)
0
5
10
15
20
25
30
A
A
A
A
(g)
Nectar consumed (ml)
0.0
0.2
0.4
0.6
0.8
A
AB
AB
B
(h)
0.0
0.2
0.4
0.6
0.8
A
A
A
A
Clivia
Salvia
Babiana
Penstemon
Clivia
Salvia
Babiana
Penstemon
Model type
Model type

Figure 2.

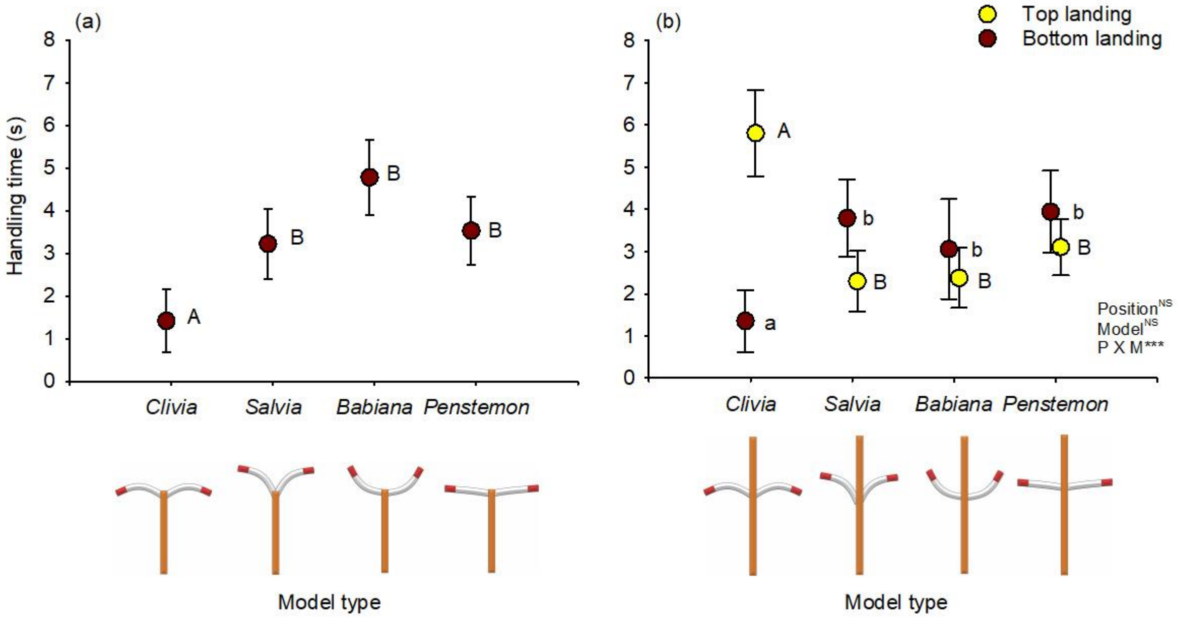
(a)
Handling time (s)
0
1
2
3
4
5
6
7
8
A
B
B
B
Clivia
Salvia
Babiana
Penstemon
Model type
(b)
Top landing
Bottom landing
A
b
B
b
B
b
B
a
Position^NS
Model^NS
P X M***
Clivia
Salvia
Babiana
Penstemon
Model type

Figure 3.

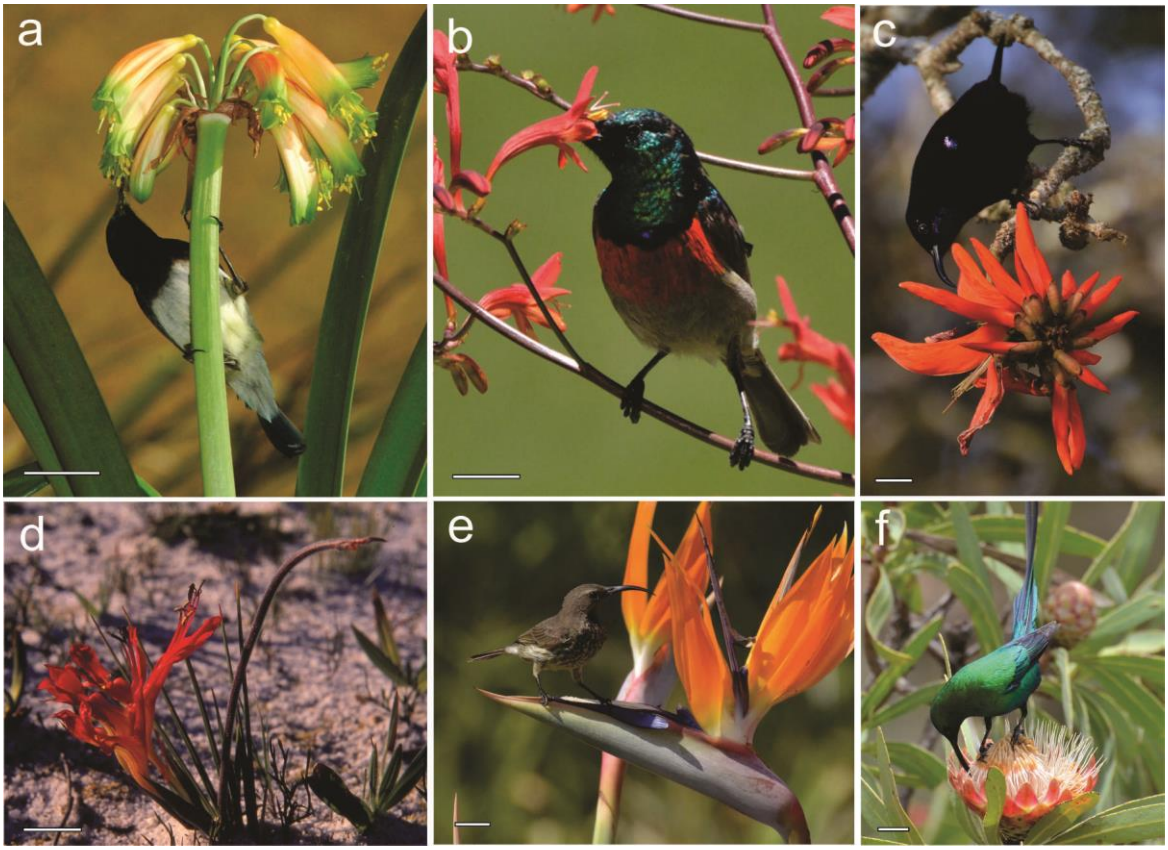

Figure 4.