THE ROLE OF MUTUALISMS IN THE EVOLUTION OF FLOWER AND FRUIT TRAITS IN THE HAEMANTHINAE (AMARYLLIDACEAE)

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ABSTRACT

Diversification of flower and fruit traits are distinctive features of angiosperm radiation. Evolutionary shifts between different animal pollinator groups are usually accompanied by modifications in flower traits. Similarly, shifts between different animal seed dispersers are usually accompanied by modifications of fruit traits. The aim of this study was to assess the functional importance of flower and fruit traits in the African sub-tribe Haemanthinae (Amaryllidaceae) which consists of the closely related genera *Scadoxus* and *Haemanthus*. These genera occur in multiple habitats and exhibit a diversity of floral and fruit traits that are potentially related to their pollination and seed dispersal systems which has not been previously studied.

The species of *Scadoxus* can be categorized according to two types of inflorescence architecture – 'paintbrush', where the reproductive parts of the flowers are tightly packed together, or 'open brush', where the reproductive parts are widely spaced. All *Haemanthus* species have 'paintbrush' inflorescences. I investigated the functional significance for pollination of these two inflorescence types. The genus *Scadoxus* appears to have undergone several shifts from butterfly to bird pollination. I found that both subspecies of *S. multiflorus* with open brush inflorescences are pollinated by butterflies and that *S. puniceus* and S*. membranaceus* with paintbrush inflorescences are pollinated by sunbirds. The system of butterfly pollination involves pollen being transferred from plant to plant via the surface of the butterfly's wings. This system, previously thought to be unusual, is apparently common in the South African Amaryllidaceae and I speculated that nine species are pollinated this way. I found that *S. multiflorus* subspecies *katherinae* displays a system of late-acting self-incompatibility, whereby the tubes of self pollen are stopped at the ovary, as shown previously for other Amaryllidaceae. Self-incompatibility was also found for the sunbird pollinated *S. puniceus*. Intriguingly, *S. membranaceus*, which is very similar in appearance to *S. puniceus*, but rarely visited by sunbirds in its coastal forest habitat, was found to be self-compatible and capable of autonomous seed production.

The genus *Haemanthus,* a sister clade to *Scadoxus,* occurs only in South Africa and Namibia, and consists entirely of species with 'paintbrush' style inflorescences. *Haemanthus deformis* is geoflorous with a very short peduncle and is pollinated by sunbirds that stand on the ground next to the inflorescence and bend over to feed on the nectar in the flowers. In the closely related *H. albiflos*, the inflorescence stem is longer and used as a perch. Both species have white flowers which is unusual for sunbird-pollinated plants. *Haemanthus coccineus* is found in the Cape Floral Region and has red flowers and bracts. This species has a much longer peduncle and is pollinated by sunbirds which grip onto the peduncle or bracts when feeding. *H. humili*s subsp. *hirsutis* is also visited by sunbirds which use the long peduncle as a perch when feeding on the pink flowers. Selective exclusion experiments indicated that *H. humili*s subsp. *hirsutis* is pollinated by both birds and insects, while *H. coccineus* and *H. deformis* are reliant on sunbirds.

The tribe Haemantheae is defined by having fleshy, brightly coloured baccate fruits with large, recalcitrant seeds. No other species in the family have such a fruit type and the closest related tribe, Amaryllideae, have fruits characteristic of abiotic dispersal. *S. multiflorus* subsp. *katherinae* and *S. puniceus* occur in similar habitat of coastal to inland forested vegetation. I found that seeds of both taxa are dispersed by monkeys, which eat the fruits, depulping the seeds, and then spitting them out. In the genus *Haemanthus*, fruits are softer, and many species occurs in habitats without monkeys. I found that seeds of *H. deformis* are dispersed by birds and rodents which either depulp the seeds right next to the plant or disperse the seeds further away by carrying the fruits elsewhere.

The seedlings require a shady microhabitat in bushclumps for survival and the dispersal system appears to favour either short distance dispersal into the immediate bushclump habitat or longer distance dispersal to different bushclumps.

In conclusion, inflorescence and flower structure in the subtribe Haemanthinae play key roles in different pollination systems, with flowers in the paintbrush style inflorescences of *Scadoxus puniceus* and several *Haemanthus* species being pollinated by sunbirds, and flowers in open brush style inflorescence of *S. multiflorus* being pollinated by butterflies. Furthermore, the fruits of Haeminthinae are shown to be specialised for frugivory by various animals which discard the recalcitrant seeds. Mutualisms between various animals in Haemanthinae have therefore had an important impact on the evolution of flowers and fruit traits in this amaryllid subtribe.

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of Kwa-Zulu Natal, Pietermaritzburg, from January 2020 to October 2022 under the supervision of Professor Steven D. Johnson.

This thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

[redacted]

HANNAH C BUTLER

October 2022

I certify that the above statement is correct

Professor Steven D. Johnson (supervisor)

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

DECLARATION 1 - PLAGIARISM

I HANNAH CLAIRE BUTLER declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a) their words have been re-written but the general information attributed to them has been referenced;

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DECLARATION 2 - PUBLICATIONS

Details of contribution to publications that form part of and / or include research in this thesis.

Publication 1 (Chapter 2)

Butler, H.C., Johnson, S.D., 2020. Butterfly-wing pollination in *Scadoxus* and other South African Amaryllidaceae. Botanical Journal of the Linnean Society 193, 363-374.

Publication 2 (Chapter 3)

Butler, H.C., Cozien, R.J., Johnson, S.D., 2022. For the birds? Contrasting pollination and breeding systems of the paintbrush lilies *Scadoxus puniceus* and *S. membranaceus* (Amaryllidaceae). Plant Systematics and Evolution 308.

Publication 3 (Chapter 5)

Butler, H.C., Johnson, S.D., 2022. Seed dispersal by monkey spitting in *Scadoxus* (Amaryllidaceae): Fruit selection, dispersal distances and effects on seed germination. Austral Ecology 47, 1029-1036.

Chapters 4 and 6 of this thesis are mansucripts which have either been submitted or are intended for submission to appropriate journals. In all cases, author contributions are as follows:

HCB and SDJ conceived the study. HCB collected and analysed the data with assistance from SDJ. HCB wrote the manuscripts with inputs from SDJ.

I certify the above statements are correct

[redacted]

HANNAH C BUTLER

Steven D. Johnson (supervisor)

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CHAPTER 1: THESIS INTRODUCTION

Pollination

Organisms evolve by becoming adapted to their environment, and plant-animal interactions have been studies for centuries. Pollination biology really gained traction post-Darwin and has resulted in a still expanding field (Darwin, 1859; Faegri and van der Pijl, 1979). Flowering plants show remarkable adaptations to their pollinators which has led to angiosperm diversification (Lloyd and Barret, 2011; van der Niet and Johnson, 2012). Pollinators are widely used by flowering plants to facilitate the transfer of pollen and these interactions can be an important part of ecosystem functioning (Albrecht et al., 2012). If a plant uses biotic pollinators, its flowers (or cones in the case of gymnosperms) perform several key functions: to attract a pollinator; place a high quantity of pollen onto the pollinator; and to receive high quantities of high quality pollen from the pollinator (Barrett and Harder, 2017; Harder and Wilson, 1994). A flower's morphology thus evolves to accommodate a pollinator's morphology as well as to manipulate pollinator behaviour (Armbruster et al., 2009a; Johnson and Steiner, 2000; van der Niet et al., 2014a). Critical steps for understanding pollinator-driven evolution are to determine a flower's morphology (as well as other traits), then to determine the function of these traits in relation to morphology and behaviour of pollinators, and then to interpret these traits in light of the known phylogenetic relationships among species (Armbruster et al., 2009b).

The mechanical fit between a flower and its pollinator and the associated advertising and reward traits are such that different plant species have evolved similar suites of traits, called pollination syndromes, reflecting common solutions to utilise the same pollinator, (Faegri and van der Pijl, 1979). Whilst they are useful, pollination syndromes are often an over-simplification of the huge array of floral diversity (Ollerton et al., 2009). Generally, pollination syndromes are better understood when functional groups rather than individual species are seen as the visitors (Fenster et al., 2004). A vast difference in visiting functional groups requires experimentation to find out the 'true' pollinator; or the functional group that drives floral evolution the most. Mechanical fit between pollinator and flower is required to export and import as much pollen of the same species as possible (Stebbins, 1970). If a flowering species best suits a functional group of pollinators, then it is that functional group that most affects the evolution of the flowering species. Thus, pollination shifts, or even divergent use of the same pollinator can result in speciation.

Studying the pollination system of a single species gives a limited view in terms of macroevolution, or the processes that result in the evolution of new taxonomic groups such as species (van der Niet and Johnson, 2012). For a fuller understanding of the evolutionary history involved in divergence, several related species should be examined. Studies that have been done on entire genera or even families show just how important pollination systems are in understanding the evolution and diversity of flowering plants (Borg-Karlson, 1990; Grant and Grant, 1948; Kiepiel and Johnson, 2014b; Manning and Goldblatt, 2005; Ollerton et al., 2019; Paules, 2006; Taylor and Williams, 2009). These indicate that diversification between species often correlates with shifts among different pollinators (Johnson, 2010; van der Niet and Johnson, 2012). Speciation can result from evolutionary floral modifications driven by different pollinators (Kiepiel and Johnson, 2014a). Such adaptations can result in reproductive isolation, further facilitating the process of speciation. The evolution of such isolation can be used to help delimit species (Johnson, 2006; Peter and Johnson, 2014).

The influence of inflorescence structure on pollination

Floral structure influences the way that a pollinator interacts with the flower and impacts what type of pollinator can access the rewards of the flower (Fenster et al., 2004). This is also the case for inflorescence structure, although this is less frequently studied and understood (Wyatt, 1982). Inflorescences may utilise the foraging behaviour of their pollinators to decrease geitonogamous self-pollination which has deleterious effects even in species that are self-incompatible.

An inflorescence with many flowers that have few ovules often functions as a single blossom (for example, many members of the Asteraceae family). Thus, the flowers of a larger inflorescence can be viewed not individually but as a collective (Burtt, 1961). The function of a larger inflorescence is to increase pollinator attractiveness, so that the larger the inflorescence the higher the visitation rate (Harder et al., 2004). There is a direct conflict in inflorescences between increasing visitation through attractiveness and increasing self-pollination which can come with costs for offspring quality as well as ovule and pollen discounting (Harder et al., 2004). Umbels increase the amount of pollinator-mediated self-pollination the most as compared to other inflorescences (Harder and Jordan, 2006). Competition among ovaries appears to be a key limiting factor when it comes to umbel fruit set (Wyatt, 1980).

Whilst inflorescence structure is influenced by pollinator behaviour, the inflorescence structure also influences pollinator behaviour. ‘Paintbrush’ inflorescences are umbels with many densely packed co-sexual flowers. A paintbrush style of inflorescence is not clearly associated with any of the pollination syndromes but rather appears to attract a range of visitors. However, many studies have shown individual plant species with paintbrush inflorescences to be pollinated by very specific pollinator groups. For example, the genus *Protea* is pollinated by a variety of pollinator groups, including insects, non-flying mammals, and birds (Biccard and Midgley, 2009; Collins and Rebelo, 1987; Steenhuisen and Johnson, 2012).

Specialization in pollination has often driven speciation and is facilitated by floral structure, which either attracts only a certain visitor or deters others (Anderson et al., 2014). This may be in the form of size, scent, colour, or reward type, as well as plant habit and habitat. For example, *Protea* flowers may be very specialised, using traits such as different scent compounds which have evolved to increase specificity in pollinator groups (Steenhuisen et al., 2012a). When many kinds of visitors are nonetheless attracted, certain floral traits may inhibit certain visitors from contributing to pollination. Tube length for example, in individual flowers or in inflorescences, can act as a filter for certain pollinator groups (Anderson et al., 2014). Individual flowers of inflorescences may therefore be a better indicator of the plant’s pollination system than the inflorescence structure itself.

Bird versus insect pollination

Although less common than insect pollination, bird pollination occurs in 3-5% of species in floras in the southern hemisphere (Johnson, 2022). Despite this, bird pollination in African systems have largely gone unstudied (Janecek et al., 2022; Nsor et al., 2019). Work in South Africa have shown that African honeybees often make a poor substitute and can disrupt bird pollination systems (Hargreaves et al., 2012; Vaughton, 1996). Paternal diversity is increased with bird pollination as compared to insect pollination (Krauss et al., 2017). This is due to birds travelling further between plants, not grooming extensively, trap-lining, and visiting only particular species during a foraging bout (Stiles, 1978). Birds are territorial and are also dictated by their landscape of fear, which interrupts their feeding bouts, ensuring that the bird moves between many plants and rapidly. Birds are also more dependable pollinators because of their continuous need for nectar (Stiles, 1978).

Shifts from bee to bird pollination have occurred multiple times in multiple plant families and occur more often than shifts from bird to bee pollination (van der Niet and Johnson, 2012). Many of these shifts are due to a change in traits that favour bird pollination and discourage bee pollination (Castellanos et al., 2004). Any shift from one pollinator to another comes with a distinct shift in traits that matches the ‘new’ pollinator, and the same is true for the shift from bee to bird pollination. Traits that have evolved to facilitate specialist bird pollination include nectar as a reward, orientation of the flower, lack of odour, and tube length (Faegri and van der Pijl, 1979; Johnson and Nicolson, 2008; Stiles, 1978). For example, in *Protea*, shifts appear to have been from bird to insect, with scent being the main driver in the change (Steenhuisen et al., 2012a).

Relatively small changes in traits, such as an increase in tube length, can limit the possibility of bee pollination and increases the likelihood of specialist bird pollination (Cronk and Ojeda, 2008). The colour red is often associated with bird pollination and is thought to deter bee visitors (Raven, 1972). Most often, plants evolve from generalists to specialist forms of pollination (although there are many exceptions) (Stebbins, 1970). Honeybees, for example, are generalists that visit a huge variety of flowering plants to collect various rewards, such as nectar, oil, and pollen but are generally less effective pollinators than birds (Page et al., 2021). Diller et al. (2022) showed that honeybees deposited lower quality self-pollen for a species of *Aloe* and suggested that this was due to aspects of foraging behaviour such as lack of movement between plants by the honeybees.

Identifying effective pollinators

Observing animals interacting with plants can be challenging on account of the behaviour of animals, as some are nocturnal, elusive, or rare. Direct observations by humans can also be time-consuming and may frighten off animals. One method now used extensively to at least supplement and improve human observations for determining flower visitors is camera trapping (Krauss et al., 2018; Steen, 2017). Most commercially available camera traps use passive infrared sensors to detect movement based on temperature changes in the environment in its field of view (Swann et al., 2004). This triggers photographs or videos to be taken, recording whatever has caused the trigger. Depending on the subject matter and purpose, the cameras can be set at various distances, for example, a shorter distance can be used to capture the activities of small pollinating insects (Ortmann and Johnson, 2020).

Ortmann and Johnson (2020) showed that a range of animals and their interactions can be recorded using camera traps, with 80% of rodents being recorded at one metre, and 80% of birds being recorded at 600mm. This figure dropped dramatically with distance, so that no observations of birds were recorded when cameras were set at four metres. Camera trapping can also be used to determine more about the interactions themselves, such as time of visit, bout duration and potential fitness consequences (Cozien et al., 2019; Johnson et al., 2019a). For example, van der Niet et al. (2014b) used camera trapping to determine whether a visitor was a potentially legitimate pollinator or a nectar thief, which pierce a hole at the base of a floral tube, drinking the nectar and bypassing the reproductive organs. Truly legitimate pollinators transfer pollen and the presence of pollen on animal bodies can be visible on camera trap footage (Johnson and Van der Niet, 2019; Melidonis and Peter, 2015).

Many plant species show a generalist system of pollination based on the number of floral visitors (Waser et al., 1996). Visitation rates however can be an inaccurate representation of what is the most effective pollinator, or the pollinator that has the most impact on plant reproductive fitness (Stebbins, 1970). Pollinator syndromes, or suites of traits found in flowers associated with a particular pollinator functional group, have been interpreted as the outcome of selection by the most effective pollinator (Faegri and van der Pijl, 1979; Fenster et al., 2004). Secondary pollinators may disrupt the evolution of floral traits; however, the most effective pollinator generally does fit and define the pollination syndrome (Johnson and Wester, 2017; Rosas-Guerrero et al., 2014).

Determining the most effective pollinator can be complicated. Generally, it requires knowing the impact on seed set which is determined by the kind and amount of pollen deposition from each type of visitor (Ne'eman et al., 2009). To ascertain this, single visit deposits or the amount of pollen from the study species found on a visitor need to be established (King et al., 2013). This can be difficult when dealing with larger visitors, such as birds, which cannot be easily caught, or with larger inflorescences that are difficult to manipulate, or with pollinators that are shy and cannot easily be observed.

One of the simpler ways to determine the impact that each visitor type makes is by using exclusion cages. These cages can be constructed from mesh with apertures that exclude larger visitors, such as birds, but allow smaller visitors, such as honeybees to visit legitimately (Botes et al., 2009; Vaughton, 1996; Waser, 1979; Wester and Johnson, 2017). These are placed around the flower or inflorescence from the bud stage until the fruiting stage. One can then compare the fruit and seed set by the smaller visitors to that set by all visitors (a natural control) which includes the larger visitors. This gives a direct indication of at least which size of visitor contributes more to plant reproductive fitness (Botes et al., 2009). Caging experiments have been used by many to ascertain the importance of birds versus insects to pollination (Ratto et al., 2018). In *Protea*, for example, cages revealed that certain species are dependent on insect pollination while others were not (Hargreaves and Johnson, 2004; Steenhuisen et al., 2012b). Caging, however, does not always provide accurate results as larger visitors may still find a way through cages and smaller visitors may be deterred or their behaviour altered. For example, Paton and Turner (1985) found that honeybees visited caged plants more frequently than uncaged plants of *Banksia ericifolia*, and presumed this was due to caged inflorescences having more nectar.

Self-incompatibility in plants

Plant breeding systems determine the extent to which a plant is reliant on its pollinators (Grant, 1948). Generally, those species that have evolved selfing systems show a specific suite of floral characteristics that facilitate autonomous pollination and reduce their 'showiness' to pollinators. However, there are showy selfers and these appear to have a 'best of both worlds' strategy that makes use of delayed selfing for reproductive assurance (Cozien, 2021; Fenster and Martén-Rodríguez, 2007). Self-pollination, whether autonomous or pollinator-mediated has disadvantages in terms of inbreeding depression in progeny as well as pollen and ovule discounting.

Self-incompatibility, where pollen from the same individual is rejected by the female, is common and has evolved multiple times in angiosperms (Allen and Hiscock, 2008). There are several types of self-incompatibility which involve different sites and timing of rejection (Barrett, 1988). Understanding the genetic basis of these has been of much interest for many years (Takayama and Isogai, 2005).

The type and significance of self-incompatibility is important in determining the effect this may have on overall reproductive success.

Late-acting or ovarian self-incompatibility (LSI), where self-pollen is rejected following penetration into the ovule, is surprisingly common for such an 'expensive' form of self-incompatibility (Gibbs, 2014). Research done on South African species of Amaryllidaceae have shown systems of LSI. Notable examples include *Clivia gardenia* and *C. miniata* (Kiepiel and Johnson, 2014a), *Cyrtanthus breviflorus* (Vaughton et al., 2010), and *Cyrtanthus contractus* (Johnson et al., 2019b). The European amaryllid genus *Narcissus* shows a range of self-compatibility, from LSI to full self-compatibility (Baker et al., 2000; Barrett et al., 2004; Cesaro et al., 2004; Sage et al., 1999; Simón-Porcar et al., 2015).

In LSI systems where ovules reject self-pollen tubes, ovules are wasted, reducing the opportunity for cross fertilization (Barrett et al., 1996). Essentially, male action inhibits female reproductive success, causing sexual conflict. One way to avoid or at least mitigate ovule discounting caused by LSI is pre-potency, where self-pollen tubes are slower to reach the ovules than cross-pollen tubes. This was not, however, found for *Cyrtanthus contractus*, where a mix of self-pollen and cross-pollen still resulted in a very low seed set and self- and cross-pollen tubes took the same amount of time to penetrate the ovules (Johnson et al., 2019b).

Seed dispersal mechanisms

Frugivore-plant interactions took longer to develop into a field of its own as compared to pollination biology, with important work done only in the late 20th century (Janzen, 1970; Levey et al., 2002). Seed dispersal is now understood to be important in maintaining and renewing plant communities. Much like pollination syndromes, seed dispersal mechanisms are associated with a suite of fruit and seed characteristics (Gautier-Hion et al., 1985). For example, the monkey-bird seed disperser hypothesis, as described by Gautier-Hion et al. (1985), shows partitioning of the group from others, such as the ruminant-rodent syndrome, and includes fleshy, brightly coloured fruits with no protective seed cover.

Modifications for seed dispersal have contributed to the radiation of plants (Herrera, 1989; Howe and Smallwood, 1982). For example, plants have evolved several adaptations to zoochory, such as the evolution of a reward of edible, fleshy, and brightly coloured fruit (Gautier-Hion et al., 1985; Snow, 1981). Frugivore-plant mutualisms are common, and plant-frugivore interactions have promoted evolution in both plants and the frugivores (Eriksson, 2016; Gómez and Verdú, 2012). Plant-frugivore co-evolution is however often considerably more diffuse than plant pollinator co-evolution.

The role that frugivores play in seed dispersal includes fruit-processing. Frugivores manipulate fruits and seeds in many ways, such as dropping, masticating, swallowing and defecating, regurgitating or spitting (Lambert, 2002). Fruit processing has an impact on the dispersal distance and post-dispersal factors such as germination and seedling recruitment (Lambert, 2002; Rowell and Mitchell, 1991). Fruit pulp for example may inhibit germination, so that the removal of pulp by a frugivore increase germination ability and therefore the impact of seed dispersal (Chimera and Drake, 2010).

Recalcitrant seeds are desiccation sensitive and germinate and establish rapidly (Berjak et al., 1989). Desiccation sensitive seeds tend to be larger to store more water and nutrients and are more common in wetter environments and often germinate better in shade (Tweddle et al., 2003;

Vazquez-Yanes and Orozco-Segovia, 1993). A lack of desiccation tolerance constrains the seed to being dispersed only to environments where the plant will germinate and be reproductively successful (Pammenter and Berjak, 2000). The dispersal biology of plants with recalcitrant seeds has not been well understood. Some species of Amaryllidaceae have fleshy fruits containing toxic recalcitrant seeds which are dispersed when they are discarded without ingestion by frugivores (Kiepiel and Johnson, 2019).

Directed seed dispersal, or where seeds are dispersed disproportionally to suitable sites for the seeds, is obviously advantageous for plants where germination and seedling recruitment may perform better only in certain microhabitats (Wenny, 2001; Briggs, Vander Wall & Jenkins, 2009). The distance of dispersal is therefore critical in ensuring seedling survival, not only in dispersing the seed further than the cramped conditions of the parent plant, but also dispersing the seeds to new suitable habitats, increasing colonization as well as genetic variability.

Study system

The family Amaryllidaceae shows remarkable variation in floral and fruit characteristics and has garnered much attention by ecologists and taxonomists for centuries (Ito et al., 1999; Meerow and Snijman, 2006). While some taxa, such as the genus *Narcissus*, have been extensively researched (Baker et al., 2000; Barrett and Harder, 2005; Barrett et al., 1996; Simón-Porcar et al., 2022), this has not been the case for African Amaryllidaceae. This global family therefore offers an opportunity to understand the reproductive ecology of plants and its role in trait diversification.

This thesis focuses on two amaryllid sister genera, *Haemanthus* and *Scadoxus* which make up the subtribe Haemanthinae in the tribe Haemantheae (Friis and Nordal, 1976; Meerow and Clayton, 2004). This tribe includes 6 genera with intriguing floral and fruit morphologies (Figure 1, 2). All species have coloured, fleshy fruits.

Scadoxus Raf. is a genus of nine species found throughout the east coast of Africa and into southern Arabia (Nordal and Duncan, 1984). The currently recognised species of *Scadoxus* that occur in South Africa are *Scadoxus multiflorus* subsp. *katherinae* (Baker) Friis & Nordal, *S. multiflorus* subsp. *multiflorus* (Martyn) Raf., *S. membranaceus* (Baker) Friis & Nordal and *S. puniceus* (L.) Friis & Nordal. *Haemanthus* L. is found solely in southern Africa and occur along the eastern and western coasts, with a few species occurring further inland (Snijman, 1984). The reproductive ecology of *Scadoxus* and *Haemanthus* has not been previously investigated despite extensive taxonomic revision of the group (Meerow and Clayton, 2004; Snijman, 1984).

Snijman (1984) speculated that habitat isolation has been the main contributing factor to speciation within the Haemanthinae. Floral differences however indicate that pollinators have been potential drivers in speciation. Of the three *Scadoxus* species found in South Africa, *S. multiflorus*, has a lax umbel type inflorescence while the other two species, *S. puniceus* and *S. membranaceus*, have a 'paintbrush' style inflorescence, which is made up of tightly packed co-sexual flowers (Duncan et al., 2017). Recent phylogenetic work on *Scadoxus* by Bødker (2020) shows that floral and inflorescence structures are highly labile in the clade.

The genus *Clivia*, closely related to *Scadoxus* and *Haemanthus* and in the tribe Haemantheae, shows floral specialization for either bird or butterfly pollination and therefore a pollinator shift which has resulted in speciation (Kiepiel and Johnson, 2014b). This shift resulted in a change in floral traits, producing flowers modified for butterfly pollination. Kiepiel and Johnson (2014b) used direct observations, camera trapping and caging experiments to determine whether butterfly or bird visitations were more important to reproductive success for various *Clivia* species. For paintbrush inflorescences such as those found for some *Scadoxus* and for *Haemanthus* species, camera trapping and caging experiments may be of particular importance as flowers are arranged in this type of inflorescence are known to attract and be pollinated by several different visitors, such as insects, birds, and non-flying mammals (for example *Protea*: (Biccard and Midgley, 2009; Hargreaves and Johnson, 2004; Steenhuisen et al., 2012b)).

All species in the tribe Haemantheae have berry-like (baccate) fruits which have a colourful exocarp and a succulent pulp surrounding desiccation sensitive seeds. Snijman (1984) speculates that either the lengthening of the peduncle or the movement of seeds along seasonal watercourses are the more likely mechanisms for dispersal of the seeds of *Haemanthus*. This was a curious assertion, given the fleshy, brightly coloured fruits which indicate a frugivore mutualism (Gautier-Hion et al., 1985). The only work that has been done on the seed dispersal of the group is that for *Clivia*, which showed that seeds of species in the genus are dispersed via monkey-spitting (Kiepiel and Johnson, 2019). The fruits of *Scadoxus* are red or orange may be dispersed in a similar way to *Clivia* given the similarities fruit and seed structure. Many *Haemanthus* species occur in areas, such as fynbos or savanna habitats, that are not frequented by monkeys. It was therefore considered possible that the seed dispersers of *Haemanthus* differ from those of *Scadoxus* and *Clivia*.

Thesis Outline

The aim of this thesis is to determine the pollination, breeding, and seed dispersal systems of the South African species of *Scadoxus* and selected species of *Haemanthus*. In chapter 2, I investigate the butterfly-wing pollination system of *S. multiflorus*, describing floral adaptations to these pollinators, and review other South African Amaryllidaceae that have been shown to share the same butterfly-wing pollination system or which could be likely candidates for this system. I also describe the late-acting self-incompatibility system of *S. multiflorus* subsp. *katherinae*. In chapter 3, I examine the pollination systems of the other two South African species of *Scadoxus*, *S. puniceus* and *S. membranaceus*, describing the main pollinators which, using camera trapping and using exclusion cages, I found to be sunbirds. I describe experiments to test if larger visitors, such as sunbirds, contribute more to reproductive success than smaller visitors. I also investigated and compared the breeding systems of both species to show the species' adaptations to their habitat. In chapter 4, I describe the use of exclusion caging and observations to investigate the importance of sunbird pollination in four species of *Haemanthus*, *H. coccineus*, *H. deformis*, *H. albiflos,* and *H. humilis* subspecies *hirsutis*. In chapter 5, I examine the seed dispersal system of *S. multiflorus* subsp. *katherinae* and *S. puniceus*, which both occur in a similar habitat, describing the monkey spitting behaviour that results in successful seed dispersal. In chapter 6, I describe dispersal of seeds of *H. deformis* by bird and rodents and investigate the implications of fruit selection and directed seed dispersal system for seedling establishment and seedling survival. Finally, in chapter 7, I summarize my results of five chapters, their importance and relevance to our current understanding of reproductive ecology in general and the evolution of the Amaryllidaceae in particular.

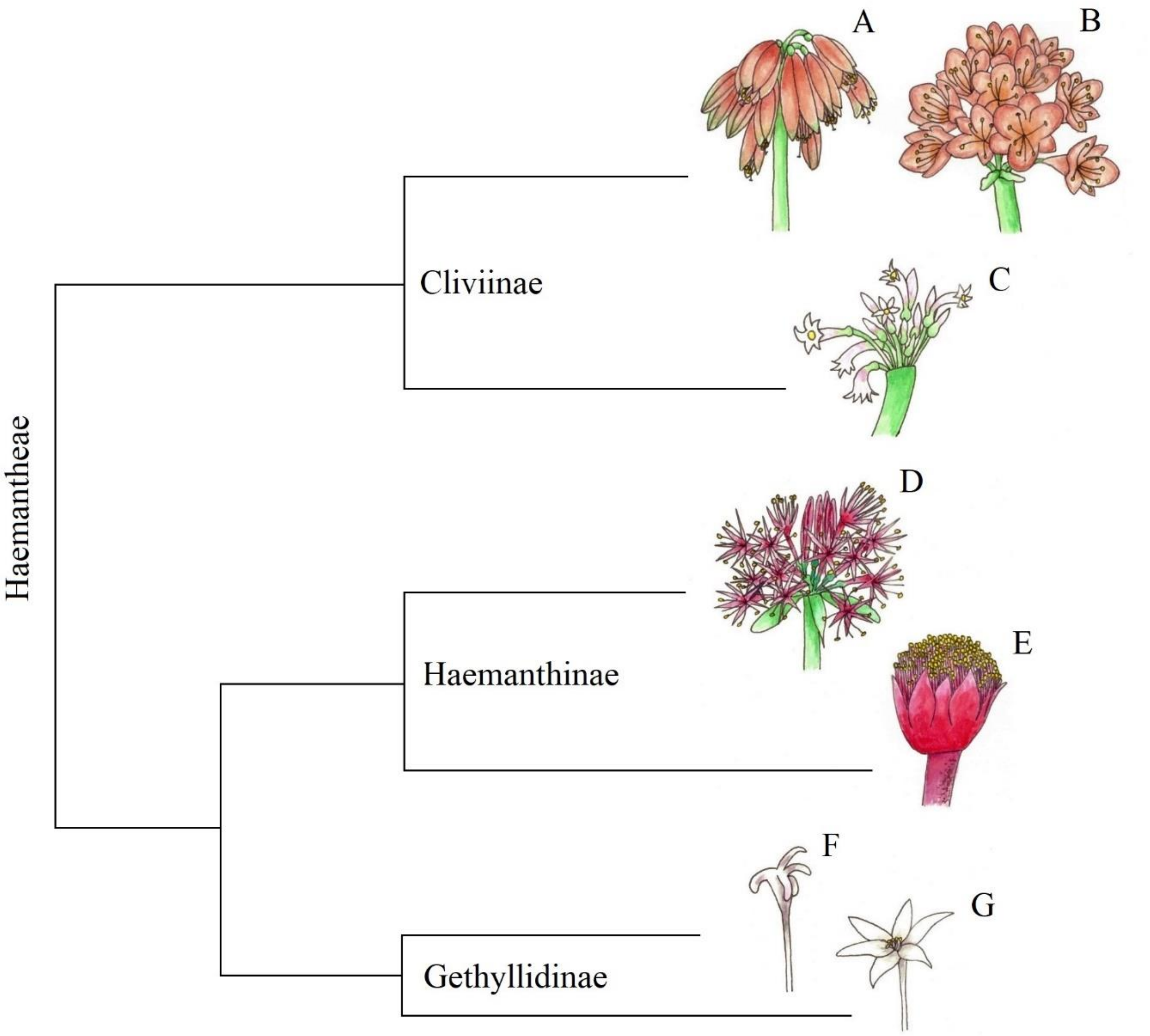

Figure 1. The flowers or inflorescences of the tribe Haemantheae (phylogeny as per Meerow and Clayton (2004)). A) *Clivia nobilis*; B) *Clivia miniata*; C) *Cryptostephanus vansonii*; (D) *Scadoxus multiflorus* subspecies *katherinae*; E) *Haemanthus coccineus*; F) *Apodolirion buchananii*; G) *Gethyllis afra*. In some phylogenies, *Apodolirion* is nested within *Gethyllis*. Drawings: HC Butler.

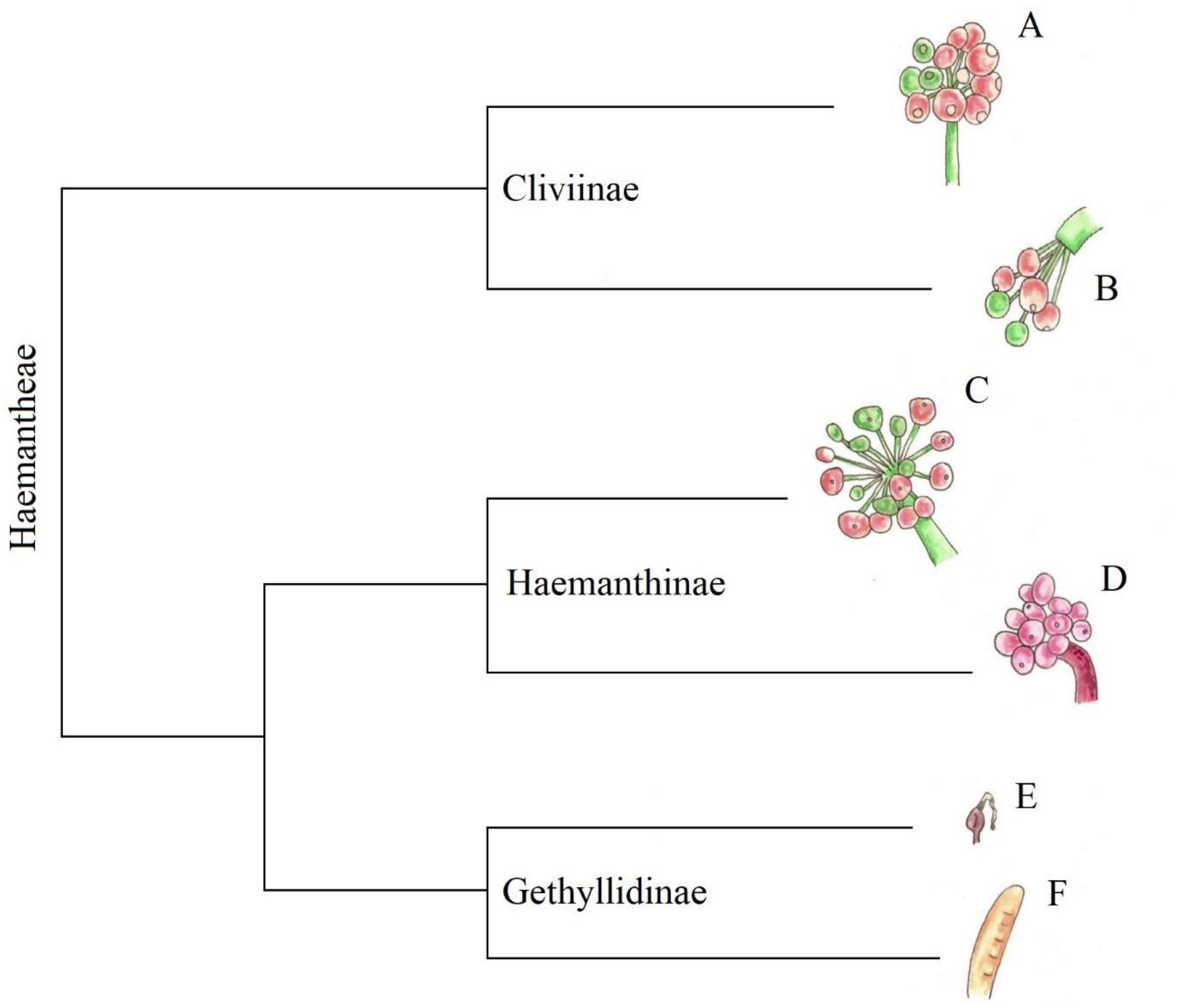

Figure 2. Fruits of the tribe Haemantheae (phylogeny as per Meerow and Clayton (2004)). A) *Clivia nobilis*; B) *Cryptostephanus vansonii*; C) *Scadoxus multiflorus* subspecies *katherinae*; D) *Haemanthus coccineus*; E) *Apodolirion buchananii*; F) *Gethyllis afra*. In some phylogenies, *Apodolirion* is nested within *Gethyllis*.

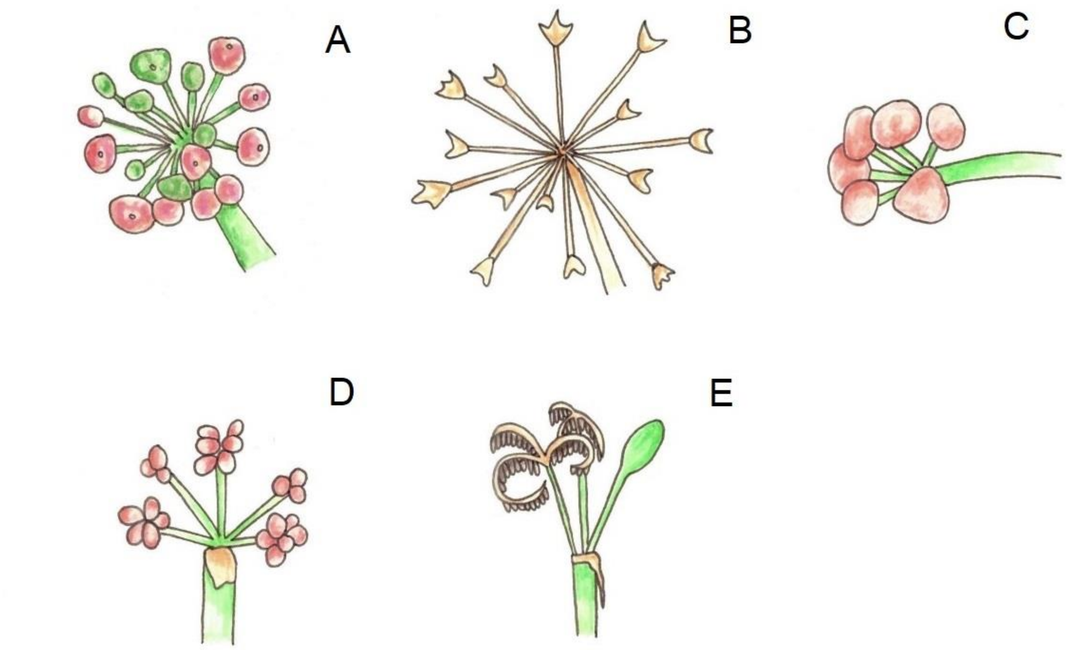

Figure 3. South African Amaryllidaceae fruits and their seed dispersal mechanism. A) *Scadoxus multiflorus* subspecies *katherinae* (zoochory); B) *Brunsvigia* (anemogeochory); C) *Crinum* (atelechory); D) *Strumaria* (autochory); E) *Cyrtanthus* (wind).

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CHAPTER 2: BUTTERFLY-WING POLLINATION IN *SCADOXUS* AND OTHER SOUTH AFRICAN AMARYLLIDACEAE

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Butterfly-wing pollination in *Scadoxus* and other South African Amaryllidaceae

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Understanding the evolution of floral morphology requires information about the identity of pollinators as well as the specific mechanisms of pollen transfer. Based on preliminary field observations and floral structure, we hypothesized that pollination mechanisms involving the transfer of pollen on butterfly wings occur in several lineages of South African Amaryllidaceae. Here we report findings from a detailed study of butterfly-wing pollination in two subspecies of *Scadoxus multiflorus* and review the prevalence of this pollination mechanism among other Amaryllidaceae in southern Africa. We established that *S. multiflorus* subsp. *katherinae* is genetically self-incompatible and thus entirely reliant on pollinators for seed production. We determined that this subspecies is pollinated almost exclusively by large swallowtail butterflies, principally males of the mocker swallowtail *Papilio dardanus cenea*. Flowers of *S. multiflorus* subsp. *multiflorus* are pollinated by pierid and swallowtail butterflies. Pollen is deposited on the ventral surface of the wings of butterflies as they flutter over the strongly exserted stamens. We predict that butterfly-wing pollination occurs in at least nine species of South African Amaryllidaceae, which may reflect several independent origins of this mechanism. The flowers of these species are red or orange with strong herkogamy and are either bowl-brush or open-brush in shape. We provide maps of the distribution of pollen on the ventral surface of the wings of pollinators for four of these species. All four appear to be pollinated via the ventral surface of large butterfly wings, with the floral structure facilitating the process. These findings illustrate the importance of investigating pollen transfer mechanisms in order to understand patterns of floral diversification and floral convergence.

ADDITIONAL KEYWORDS: pollen – pollination syndrome – self-incompatibility – single-visit pollination – specialized pollination.

INTRODUCTION

Animals are widely used by flowering plants to facilitate pollen dispersal. These plant–pollinator interactions are an important part of ecosystem functioning and are vital for outcrossing species (Ollerton, Winfree & Tarrant, 2011). If a plant uses biotic pollinators, its flowers perform several key functions: to attract a pollinator, to place pollen from the anthers onto the pollinator, and to receive high-quality pollen from the pollinator onto its stigmas (Harder & Wilson, 1994; Barrett & Harder, 2017; Minnaar *et al.*, 2018). Floral morphology plays a key role in these functions by accommodating pollinator morphology, manipulating pollinator behaviour and ensuring effective pollen transfer (Harder & Barrett, 1996; Armbruster *et al.*, 2009a). Flowers provide some of the most outstanding examples of how species become adapted to their environment and how this can lead to diversification (van der Niet & Johnson, 2012). A proper understanding of the evolution of floral morphology requires investigations of the identity of pollinators, the mechanisms of pollen transfer, the level of outcrossing and the mating system (Grant, 1948; Armbruster *et al.*, 2009b).

Butterflies use their long proboscides to visit a wide range of plants for nectar, including flowers of plants that they do not effectively pollinate (Jennersten, 1984). Plants that are specialized for pollination by butterflies often show highly variable floral morphology (Reddi & Bai, 1984). Johnson & Bond (1994) argued that the morphological variation among butterfly-pollinated flowers can be explained by pollen transfer mechanisms. For example, the flowers of most butterfly-pollinated

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*Corresponding author. E-mail: JohnsonSD@ukzn.ac.za species are salverform, consisting of a tube that opens up to a disc and typically places pollen on the proboscis or head of butterflies (Massinga, Johnson & Harder, 2005; Ferrero *et al.*, 2009). However, some butterfly-pollinated flowers are brush-like with highly exserted reproductive parts, which place pollen on the wings or body of butterflies as they inspect or settle on the flowers (here termed the 'open-brush' type) (Johnson & Bond, 1994). Others are bowl-shaped, and pollen placement occurs via the wings and body as the butterfly nestles among the anthers that line the perianth bowl (here termed the 'bowl-brush' type) (Kiepiel & Johnson, 2014). Butterfly-wing pollination has been observed or inferred in multiple plant families (see Table 1).

Scadoxus Raf. is a genus of nine species of Amaryllidaceae and is found throughout eastern coastal Africa and into southern Arabia (Nordal & Duncan, 1984). Despite being used widely in horticulture, the genus has not been investigated in terms of its reproductive ecology (Meerow & Clayton, 2004; Duncan, 2012–2013). In the 1950s, Vogel assigned floral syndromes to plants based on their floral characteristics and predicted butterfly-pollination for *Scadoxus multiflorus* (Martyn) Raf. subsp. *katherinae* (Baker) Friis & Nordal (Vogel, 1954, 2012). This taxon has open-brush type flowers which are remarkably similar to those of other amaryllids suggested by Johnson & Bond (1994) to be butterfly-wing-pollinated (Fig. 1). An image of the pierid butterfly *Colotis ione* visiting flowers of *S. multiflorus* subsp. *multiflorus* in Kenya was published by Collins & Martins (2016).

The aims of the present study were to determine whether *S. multiflorus* is pollinated via butterfly wings and to determine whether this pollen transfer mechanism occurs in other amaryllids in South Africa. We specifically investigated whether *S. multiflorus* is (1) dependent on pollinators for seed production, (2) visited by butterflies and (3) effectively pollinated by butterflies, (4) whether butterflies carry pollen on their wings, and (5) whether butterfly-wing pollination occurs in other South African Amaryllidaceae.

MATERIAL AND METHODS

STUDY SITES AND TAXA

Scadoxus multiflorus subsp. *katherinae* occurs in forested swampy habitats along the east coast of South Africa. This taxon flowers primarily in February, producing a single inflorescence per plant, each consisting of 82.17 ± 38.57 ($n = 6$ plants) red flowers that have an open-brush morphology. *Scadoxus multiflorus* subsp. *multiflorus* has similar brush-like flowers and inflorescence morphology, but it flowers in

Table 1. Plant species speculated or observed to be pollinated via butterfly wings

Family	Species	Evidence	Reference
Amaryllidaceae	*Clivia miniata* (Lindl.) Bosse	O; M; P; D; W	Kiepiel & Johnson (2014)
	Brunsvigia marginata (Jacq.) W.T.Aiton	O	Johnson & Bond (1994)
	Cyrtanthus elatus (Jacq.) Traub	O	Johnson & Bond (1994)
	Cyrtanthus guthrieae L.Bolus	O	Johnson & Bond (1994)
	Nerine sarniensis Herb.	O	Johnson & Bond (1994)
	Cyrtanthus montanus R.A.Dyer	S; M	Johnson & Bond (1994)
	Cyrtanthus flammosus Snijman & van Jaarsv.	S; M	Snijman & Meerow (2010)
	Cyrtanthus taitii G.D.Duncan	S; M	Duncan (2018)
Capparaceae	*Cadaba fruticosa* Druce	O; M	Aluro & Rao (2002)
Colchicaceae	*Gloriosa minor* Rendle	O	Martins (2014)
	Gloriosa superba L.	O; W	Hingston (1931)
Ericaceae	*Rhododendron calendulaceum* (Michx.) Torr.	O; P; D; W; E	Epps, Allison & Wolfe (2014)
Fabaceae	*Caesalpinia pulcherrima* (L.) Sw.	O; W	Cruden & Hermann-Parker (1979)
Iridaceae	*Hesperantha coccinea* (Backh. & Harv.) Goldblatt & J.C.Manning	O	Johnson & Bond (1994); Goldblatt *et al.* (2004)
	Several species of *Gladiolus* L.	O; M	Goldblatt & Manning (2002)
Lamiaceae	*Clerodendrum infortunatum* L.	O; M; W	Reddy & Reddi (1995)
Liliaceae	*Lilium martagon* L.	O; W	Corbera, Alvarez-Cros & Stefanescu (2018)
	Lilium philadelphicum L.	O	Barrows (1979); Edwards & Jordan (1992)
	Lilium humboldtii Bull.	O	Davis (1956)
	Lilium superbum L.	O; W	Adams (2015)

O = observation; S = speculation; P = pollen deposition on stigmas; D = single visit pollen deposition; M = floral and or butterfly measurements; W = pollen deposition on wings; E = large butterfly exclusion.

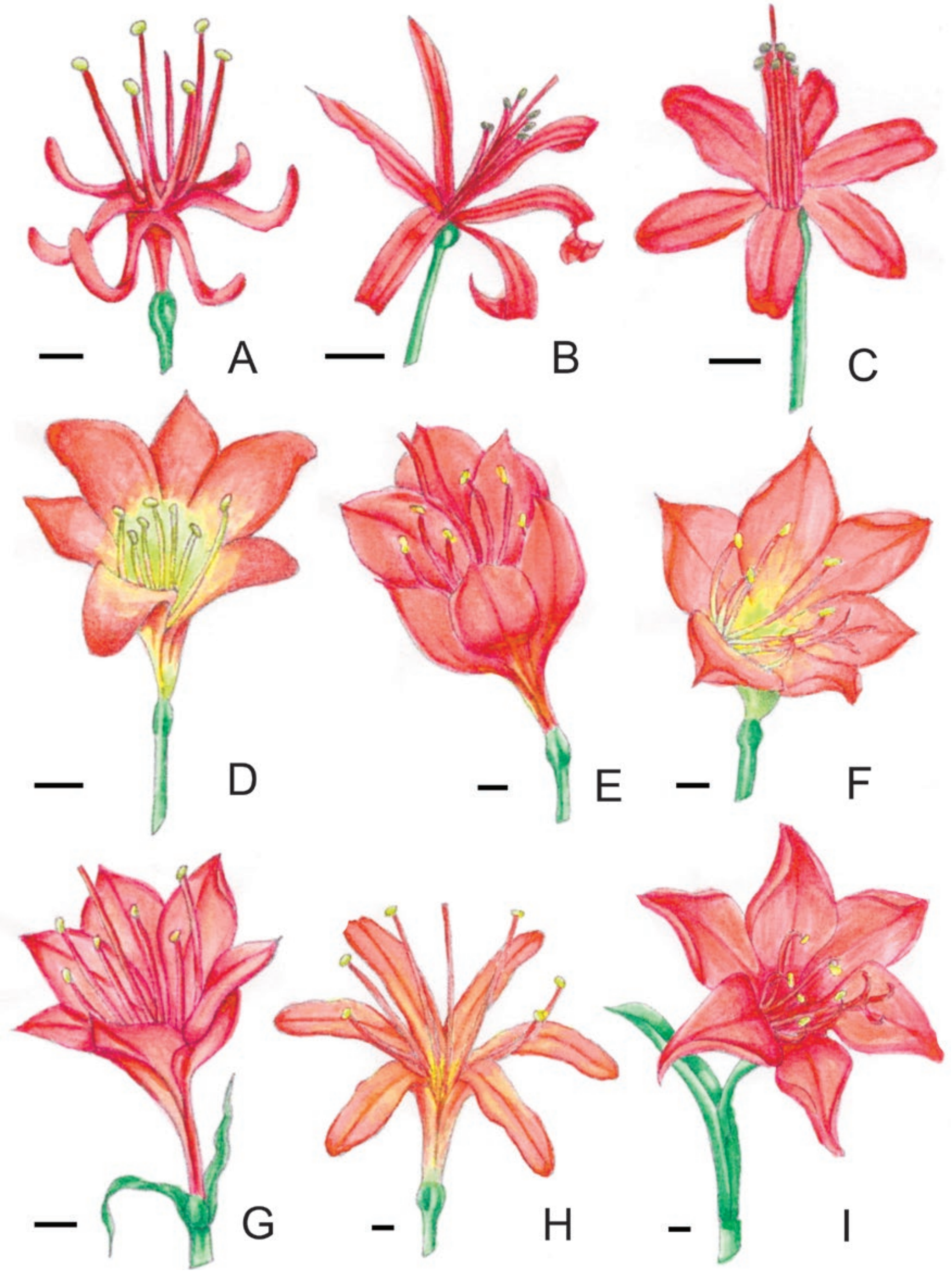

Figure 1. The flowers of South African Amaryllidaceae suspected or confirmed to be pollinated via butterfly wings. A, *Scadoxus multiflorus* subsp. *katherinae*; B, *Nerine sarniensis*; C, *Brunsvigia marginata*; D, *Clivia miniata*; E, *Cyrtanthus elatus*; F, *Cyrtanthus flamossus*; G, *Cyrtanthus guthrieae*; H, *Cyrtanthus montanus*; I, *Cyrtanthus taitii*. Scale bars: 20 mm. Illustrations: H. C. Butler.

October, occurs in savanna vegetation, and has a range extending north into East Africa.

We studied *S. multiflorus* subsp. *katherinae* in forest habitat in the Vernon Crookes Nature Reserve (30°16′17.1″″S, 30°35′35.3″E) (referred to here as VC), a large (2200-ha) natural environment, and Bendigo Nature Reserve (30°40′49.4″S, 30°29′55.6″E) (referred to here as BN), a small (*c*. 3.5-ha) forest fragment in an urban environment. We studied *S. multiflorus* subsp. *multiflorus* in savanna habitat in Ndumo nature reserve (26°56′45.7″S, 32°14′25.5″E) and at a site adjacent to the Phinda Nature Reserve (27°49′29.3″S, 32°21′48.1″E). The voucher numbers for *S. multiflorus* subsp. *katherinae* and subsp.

multiflorus are *Butler 89* and *Butler 92*, respectively (BEWS Herbarium: NU).

BREEDING SYSTEM AND FLORAL TRAITS

To test whether *S. multiflorus* subsp. *katherinae* is genetically self-incompatible, we carried out controlled hand-pollinations using either cross- or self-pollen, with both treatments being applied to *c*. 20 randomly selected flowers on each of three inflorescences picked at the bud stage and kept with their stems in water. This method was necessary because it is difficult to place exclusion bags on *S. multiflorus* inflorescences in the field without risking contact between the bag and the reproductive parts of the flower, thus leading to inadvertent self-pollination. Because of their fleshy stems, *Scadoxus* inflorescences picked at the flowering stage will produce viable seeds. Once the treated flowers had developed fruits, fruits and seeds were counted. We statistically compared the treatments using generalized estimating equations, incorporating an exchangeable correlation matrix, with plant treated as the subject to account for lack of independence among flowers treated on the same plant. For analyses of the breeding system experiment and for comparison of fecundity among populations that involved the proportion of flowers that set fruit or the proportion of ovules (each flower has three ovules) that developed into seeds, we used models that incorporated a binomial probability distribution with an events-by-trials design and logit-link function. Model significance was tested using Wald statistics due to the small sample sizes. An index of self-incompatibility (ISI) using model estimates of marginal means was calculated as 1 − selfed success/outcrossed success, where success was the mean proportion of ovules that developed into seeds. An ISI value > 0.8 is an indicator of self-incompatibility (Raduski, Hanney & Igik, 2012).

We measured, using a steel ruler, various floral parts, including stamen length, style length, herkogamy within the same flower (the distance between the stigma and nearest anther of the same flower), tube length and distance between the stigmas of consecutive flowers. Nectar volume was measured using glass micropipettes (Blaubrand, 0–5 µL) and sugar concentration was determined using a handheld refractometer (Bellingham & Stanley, 0–50%). Spectral reflectance of petals was measured using an Ocean Optics S2000 spectrophotometer, as described by Johnson & Anderson (2002). To determine whether floral traits differ between the two subspecies, statistical analysis of the morphology data was carried out using generalized linear models (GLMs) implemented in SPSS (version 26, IBM Corp.). To account for statistical non-independence of samples from the same plant, we treated plant as a subject in generalized estimating equations with an exchangeable correlation matrix. Site was treated as a fixed factor nested within subspecies. For measurement data we used models which incorporated a Gaussian distribution and identity link function. Significance was determined by Score statistics. We calculated marginal (model-adjusted) means for each subspecies.

VISITOR OBSERVATIONS AND POLLINATOR EFFECTIVENESS

The floral visitors were identified through direct observations (for *S. multiflorus* subsp. *katherinae*: 22–24 February 2018 and 12 and 21 February 2019; for subsp. *multiflorus*: 10 October 2018 and 19 October 2019), where the visitor species, activity in relation to the flowers and overall visitor frequency were recorded, typically from 09:00 to 15:00 h when butterflies were found to be most active. We captured representative individuals and measured the lengths of their proboscides, costal margin of the forewing and hindwing, the inner margin of the hindwing and the outer margin of the wing.

To assess the overall abundance and diversity of butterflies at both sites, we recorded each individual butterfly seen at the study sites. These were then identified to species level according to Woodhall (2005). For each butterfly that visited the study species, we recorded whether responses to flowers involved inspection or feeding visits. To assess the efficiency of butterflies as pollinators, we allowed butterflies to visit virgin flowers of *S. multiflorus* subsp. *katherinae* and recorded pollen deposition on stigmas. For this, two inflorescences, each with *c*. 60 flowers, were taken at the bud-stage from the field and allowed to open under laboratory conditions while the stems were in water. All the flowers on these inflorescences were then emasculated. These inflorescences were taken back to the field and placed among unmanipulated, natural inflorescences for 1 day. The identity and behaviour of visitors to these inflorescences were recorded and the number of pollen grains and butterfly scales on ten stigmas from each manipulated inflorescence were counted under a microscope (Kiepiel & Johnson, 2014).

Using different plants and without the above results, we assessed overall pollen transfer efficiency (PTE) in a population of *S. multiflorus* subsp. *katherinae* at VC in 2018 by dividing the average number of pollen grains on *c*. 60 random stigmas in the field by the average number of pollen grains removed from anthers (Johnson, Neal & Harder, 2005). To estimate pollen removal from anthers, we calculated the difference in the mean numbers of pollen grains in *c*. 15 freshly opened anthers and *c*. 25 randomly selected old anthers (each from a different flower) that had been open and exposed to visitors in the field. We placed anthers in 0.5 mL of 70% ethanol and then counted pollen grains in a 10-μL subsample placed on a microscope slide with melted fuschin gel. The number of pollen grains in each anther was then calculated by multiplying the number of counted pollen grains per slide by 50. The number of pollen grains on stigmas of the flowers that had been exposed to visitors were counted by creating microscope slides with melted fuschin gel. Estimates of total pollen production per flower, as described above, and ovule counts (each flower has three ovules) were combined to calculate a pollen to ovule ratio. We also determined natural levels of fruit set (the percentage of flowers that set fruit) in the field for both populations of *S. multiflorus* subsp. *katherinae*, using ten plants from BN and 11 plants from VC and compared natural fecundity between the populations using logistic GLMs with an events/trials structure. Significance was assessed using likelihood ratios.

POLLEN DISTRIBUTION ON BUTTERFLY WINGS

Because much of the pollen on butterfly wings is lost when they are netted and handled, we used high-resolution (16.2 mega pixels) macrophotography of butterflies visiting flowers to assess the distribution of individual pollen grains on wings of butterflies visiting flowers of *S. multiflorus* subsp. *katherinae* (*n* = 6 photographs), *Brunsvigia marginata* (Jacq.) W.T.Aiton (*n* = 5 photographs), *Cyrtanthus elatus* (Jacq.) Traub (*n* = 3 photographs) and *Clivia miniata* (Lindl.) Verschaff. (*n* = 6 photographs) (Fig. 2). The observations of *B. marginata* were conducted in a population of *c*. 400 plants at the Bainskloof pass (33°38′S, 19° 06′E) from 08:00 to 16:00 h on 8 and 9 April 2018. During this time, we observed > 20 foraging bouts by mountain pride butterflies (*Aeropetes tulbaghia*; Fig. 2A) and two foraging bouts by citrus swallowtails (*Papilio demodocus*). The observations of *Cyrtanthus elatus* were conducted in a population of *c*. 5000 plants at the Robinson Pass (33°53′S, 22°01′E) between 07:30 and 18:00 h on 22 and 23 March 2018. During this time, we observed 12 foraging bouts by mountain pride butterflies (Fig. 2B). Details of the observations of butterflies visiting flowers of *Clivia miniata* (Fig. 2C) were provided by Kiepiel & Johnson (2014). For each plant species we captured a sample of butterfly visitors and, using microscopy, confirmed that the pollen visible on their wings was from the species on which it was observed. We used wing damage and wear to identify individual butterflies in the photographs and used only one photograph per individual. A grid was placed over a basic butterfly ventral wing venation map. For each individual butterfly photographed, a score of pollen visible or not was given to each block in the grid. The percentage of butterfly individuals that scored visible pollen for a particular block was then calculated with 20% intervals and coloured accordingly. In this way, a map showing pollen distribution on the ventral surface of the wings of visiting butterflies could be created for each of the four plant species. For two butterflies (both *Papilio dardanus cenea*) captured on *S. multiflorus* subsp. *katherinae* and five butterflies [*Belenois gldica abysinnica*, *Belenois thysa thysa*, *Colotis eris eris* (two individuals), *Papilio demodocus demodocus*] captured on *S. multiflorus* subsp. *multiflorus*, we counted the total number of pollen grains on various body parts including the wings.

RESULTS

BREEDING SYSTEM AND FLORAL TRAITS

The self-pollination treatment for *S. multiflorus* subsp. *katherinae* resulted in a significantly lower mean percentage of flowers setting fruit compared to

Figure 2. Butterfly wing pollination in South African Amaryllidaceae. A, *Brunsvigia marginata* visited by *Aeropetes tulbaghia* (scale bar: 10 mm); B, *Cyrtanthus elatus* visited by *Aeropetes tulbaghia* (scale bar: 50 mm); C, *Clivia miniata* visited by *Papilio dardanus cenea* (scale bar: 10 mm). Photographs: S. D. Johnson.

the cross-pollination treatment (self: 10.34%, cross: 85.71%, $\chi^2 = 84.9$; $P < 0.0001$) and a significantly lower mean percentage of ovules in fruits that developed into seeds (self: 50.79%, cross: 66.63%, $\chi^2 = 1234.3$; $P < 0.0001$). The index of self-incompatibility for this taxon was 0.88.

The flowers of the subspecies of *S. multiflorus* differ significantly in terms of stamen exsertion, herkogamy and tube length (Table 2). Spectral reflectance revealed maximum inflection at 600 nm for *S. multiflorus* subsp. *katherinae* and 400 nm for subsp. *multiflorus* and a secondary reflectance peak in the UV region (Supporting Information, Fig. S1).

VISITOR OBSERVATIONS AND POLLINATOR EFFECTIVENESS

Scadoxus multiflorus subsp. *katherinae* was visited almost exclusively by large swallowtail butterflies (Fig. 3). Of the 14 species of butterfly observed to be present in the plant population in VC, only four were visitors to flowers of the study species (Supporting Information, Table S1). These butterflies displayed diverse behaviours in response to the flowers, sometimes only fluttering by the flowers ('inspections'), brushing the floral structures but not inserting the proboscis into the flowers; most often, however, the butterfly visitors settled to insert their proboscis and continued to flutter their wings when feeding (Video S1). Mocker swallowtails (*Papilio dardanus cenea*) were the most frequent floral visitor ($n = 18$; Table S1; Fig. 3A–C). Two pierids (*Nephronia argia*) visited the flowers and although they have a smaller wingspan than *Papilio dardanus cenea*, their wings appear large enough to still make contact with the floral reproductive parts (Table 2; Table S1). Five honey hoppers (*Platylesches moritili*) were also seen to visit the flowers but appeared too small to effect pollination (Table S1). Only three individual butterflies, all of the same species (*Hypolimnas deceptor deceptor*), were found at BN, and none of these was seen to visit the flowers at this site (Table S1). The flowers of *S. multiflorus* subsp. *multiflorus* were visited by large numbers of both pierid and papilionoid butterflies (Fig. 3E; Table 3). The smaller pierids may be effective pollinators for this subspecies as they make contact with the floral reproductive parts (Fig. 3F). A lycaenid visited (Table S1), but, given its even smaller wingspan, it is unlikely to be an effective pollinator.

The two inflorescences of *S. multiflorus* subsp. *katherinae* used for the single visit deposit experiment were both visited once by an emperor swallowtail (Fig. 3D) and once by a mocker swallowtail. After these visits, the stigmas ($n = 10$) for each inflorescence had received an average (±SE) of 77.8 ± 24.35 and 81.1 ± 26.68 pollen grains, respectively, and only one scale was found on a stigma. No pollen or butterfly scales were present on stigmas of these flowers at the commencement of the experiment.

The mean (±SE) number of pollen grains on stigmas collected from the field were not significantly different across the two populations ($\chi^2 = 3.363$; $P = 0.067$), with 9.61 [lower SE (LSE) = 9.52, upper SE (USE) = 9.68; $n = 25$] at VC and 9.76 (LSE = 9.71, USE = 9.79; $n = 35$) at BN. The mean (±SE) number of butterfly scales found on stigmas collected from the field was significantly different ($\chi^2 = 3.896$; $P = 0.048$), with 4.68 (LSE = 3.96, USE = 5.41; $n = 25$) at VC and 2.71 (LSE = 2.12, USE = 3.39; $n = 35$) at BN. PTE (i.e. the percentage of removed pollen that reached stigmas) at VC in 2018 was 1.47%. The pollen to ovule ratio was 2071.

The mean percentage of flowers that set fruit at VC in 2019 was 47.8% (LSE = 41.93, USE = 53.90). This was significantly higher than the mean of 3.33% (LSE = 2.19, USE = 5.03) at BN ($\chi^2 = 44.25$; $P < 0.0001$).

POLLEN DISTRIBUTION ON BUTTERFLY WINGS

Pollen was clearly visible on the hindwings of all six swallowtail butterfly individuals that were photographed visiting *S. multiflorus* subsp. *katherinae* (Fig. 3). Most of the pollen on the butterflies caught on this subspecies was on the ventral side of the

Table 2. Morphological and nectar traits of flowers of *Scadoxus multiflorus* subspp. *katherinae* and *multiflorus*

Trait	subsp. *katherinae*	subsp. *multiflorus*	χ^2	*P*
Herkogamy (mm)	28.19 ± 1.34 (50)	17.48 ± 1.06 (55)	13.50	<0.0001
Stamen exsertion (mm)	37.71 ± 0.90 (50)	29.34 ± 0.45 (55)	15.85	<0.0001
Style exsertion (mm)	39.76 ± 1.72 (50)	31.92 ± 0.90 (55)	9.08	0.003
Tube length (mm)	20.84 ± 0.54 (50)	12.28 ± 0.60 (55)	17.38	<0.0001
Nectar volume (µm)	2.58 ± 0.36 (50)	2.53 ± 0.43 (25)	0.01	0.926
Nectar concentration (%)	19.20 ± 0.92 (45)	23.14 ± 1.44 (23)	3.41	0.065
Distance between consecutive stigmas (mm)	31.86 ± 1.18 (50)	26.47 ± 3.08 (15)	1.74	0.188

Values are marginal (model-adjusted) means ± SE (*n*).

Figure 3. Pollinators of *Scadoxus multiflorus* with contact of butterfly wing and stigma indicated by an arrow for A, B and C. A, female *Papilio dardanus cenea* visiting *S. multiflorus* subsp. *katherinae* (scale bar: 50 mm); B, male *Papilio dardanus cenea* visiting *S. multiflorus* subsp. *katherinae* (scale bar: 10 mm); C, male *Papilio dardanus cenea* visiting *S. multiflorus*

subsp. *katherinae* (scale bar: 10 mm); D, *Papilio aphididephalus* visiting an emasculated inflorescence used to test for single visit deposits (scale bar: 50 mm); E, *Papilio demodocus demodocus* visiting *S. multiflorus* subsp. *multiflorus* (scale bar: 50 mm); F, *Belenois glidica abyssinica* visiting *S. multiflorus* subsp. *multiflorus* (scale bar: 20 mm); G, *S. multiflorus* subsp. *multiflorus* pollen on a *Belenois glidica abyssinica* wing seen under a compound microscope (scale bar 100 µm); H, *S. multiflorus* subsp. *katherinae* pollen on a male *Papilio dardanus cenea* wing seen under scanning electron microscopy (scale bar: 50 µm). Photographs: S. D. Johnson and H. C. Butler.

wing (1054 ± 473) as compared to the dorsal wing surface (95 ± 28.5) and body (49 ± 15.5 pollen grains; $n = 2$ butterflies). Pollen on butterflies caught on *S. multiflorus* subsp. *multiflorus* was also concentrated on the ventral side of the wing (1818 ± 1471.711) as compared to the dorsal wing surface (19 ± 9.54) and body (13 ± 4.56 pollen grains; $n = 5$ butterflies) (Fig. 3G).

Pollen was present on the wings of butterflies visiting the flowers of the four species of South African Amaryllidaceae investigated. Most pollen was concentrated along the veins, with a higher density and wider distribution on the hindwings (Fig. 4).

DISCUSSION

Scadoxus multiflorus is clearly specialized for butterfly-wing pollination. It is pollinated effectively by butterfly species with large wings (with a distance between the base and outer wing margin that matches or exceeds the stamen and style lengths), as evidenced by pollen placement on their wings and deposition of pollen on stigmas during single visits. The reduction of the perianth and the highly elongated filaments and styles of the brush-like flowers arranged in a compact umbel appear to be key adaptations for butterfly-wing pollination in *S. multiflorus*. The relatively flattened shape of the pollen grains may increase their adhesion to the corrugated surface created by the scales on the surface of the butterfly wing (Fig. 3H).

Butterfly visitor assemblages differed markedly between the two subspecies of *S. multiflorus*, subsp. *katherinae* being visited mainly by forest species and subsp. *multiflorus* being visited mainly by savanna species. The butterflies that visit these subspecies represent a subset of all butterflies active in their respective habitats, suggesting a degree of pollination system specialization (Supporting Information, Table S1). *Scadoxus multiflorus* subsp. *katherinae* is dependent on pollinator visits for reproduction as the breeding system assessment indicated that the taxon is genetically self-incompatible.

Butterfly-wing pollination was also recorded for three other South African species of Amaryllidaceae (*Clivia miniata*, *Brunsvigia marginata* and *Cyrtanthus elatus*). Large amounts of pollen are deposited on the ventral surface of the wings of butterflies that visit these plant species (Fig. 4). Based on similar floral morphology, we hypothesize that a further five species (*Nerine sarniensis* Herb., *Cyrtanthus flammosus* Snijman & van Jaarsv., *Cyrtanthus guthrieae* L.Bolus, *Cyrtanthus montanus* R.A.Dyer and *Cyrtanthus taitii* G.D.Duncan) are also pollinated in this manner (Fig. 1). It is therefore likely that butterfly-wing pollination has evolved several times in South African Amaryllidaceae.

Table 3. Butterfly visitors that interacted with flowers of *Scadoxus multiflorus* subspp. *katherinae* and *multiflorus*

Subspecies	Butterfly family	Butterfly species	Number of individuals
S. multiflorus subsp. *katherinae*	Pieridae	*Nephronia argia*	2
	Papilionidae	*Papilio ophidecephalus*	3
		Papilio dardanus cenea	28
	Hesperiidae	*Platylesches moritili*	5
S. multiflorus subsp. *multiflorus*	Pieridae	*Belenois glidica abyssinica*	5
		Belenois thysa thysa	8
		Colotis danae annae	4
		Colotis eris eris	3
		Colotis euippe omphale	3
		Nepheroni argia	2
	Papilionidae	*Graphium porthaon porthaon*	2
		Papilio constantinus constantinus	4
		Papilio nireus lyaeus	1
		Papilio demodocus demodocus	6
	Lycaenidae	*Aloides aranda*	1

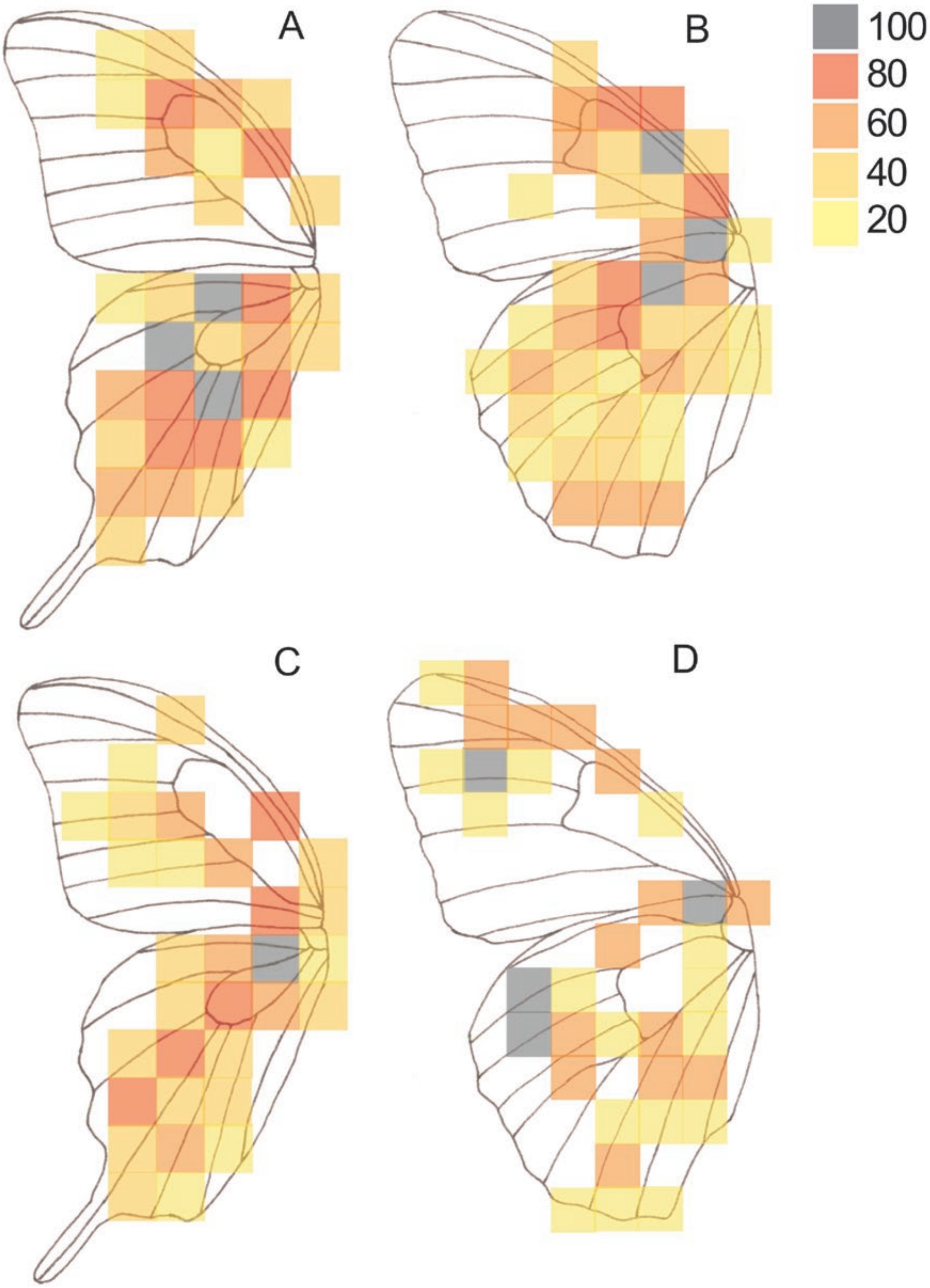

Figure 4. Pollen distribution mapped as percentages on the ventral wing surfaces of butterfly visitors. A, *Scadoxus multiflorus* subsp. *katherinae* (*n* = 6); B, *Brunsvigia marginata* (*n* = 5); C, *Clivia miniata* (*n* = 6); and D, *Cyrtanthus elatus* (*n* = 3). A and C show the wings of *Papilio dardanus cenea*, which are primary visitors of those species; B and D show the wings of *Aeropetes tulbaghia*, which are the primary visitors of those species.

This is supported by a study showing that butterfly-wing pollination in *Clivia* Lindl. was independently derived through a shift from bird pollination (Kiepiel & Johnson, 2014).

Floral traits associated with adaptations for butterfly pollination have been previously difficult to identify or formulate into a useful syndrome. For example, Vogel (1954), who produced the first modern account of pollination syndromes, amalgamated the floral traits associated with long-tongued flies and butterflies into a single, psychophilous syndrome. The classical butterfly pollination syndrome, as described by Faegri & van der Pijl (1979), characterizes the shape of butterfly-adapted flowers as salverform, with a narrow tube containing moderately concentrated, sucrose-dominant nectar and petals which form a flat surface that serves as a landing platform (Erhardt, 1990). For flowers that fit this description, such as *Pentanisia* Harv. or *Plumbago* L., the butterflies that are attracted to them probe the flowers while settled on the floral platform (Massinga *et al.*, 2005; Ferrero *et al.*, 2009). In such cases, pollen transfer is primarily via the head and proboscis of the butterfly, as this is where the interaction between flower and visitor takes place (Jennersten, 1984).

Johnson & Bond (1994), however, suggested that the morphological variation among large red and orange flowers pollinated by the butterfly *Aeropetes tulbaghia* in South Africa can be explained in terms of diverse pollen transfer mechanisms. Several species of Amaryllidaceae were identified as having a 'brush'-type flower with 'tall extended stamens projecting from a funnel-shaped flower', representing a distinct departure from the classical salverform shape of flowers. For open-brush flowers such as *Caesalpinia pulcherrima* (Fabaceae), butterflies flutter, sometimes hovering and sometimes walking on the floral platform when encountering the flowers, and continue to flutter as they extend their proboscides and attempt to drink nectar from the floral tube (Cruden & Hermann-Parker, 1979). This means their large wings constantly move over and onto the anthers and stigmas, resulting in pollen deposition on and from the wings. Of the South African Amaryllidaceae discussed here, *Scadoxus multiflorus* (Fig. 3), *Brunsvigia marginata* (Fig. 2A), *Nerine sarniensis* and *Cyrtanthus montanus* all conform to the 'open-brush' floral type. A feature of these brush-type inflorescences is that when a butterfly feeds on nectar, pollen transfer often involves contact between the wings of the butterfly and flowers that are adjacent to the flower being used as a source of nectar (Figs 2A, 3). This may explain why such flowers are presented as umbellate inflorescences in which the flowers overlap, and many flowers are usually open and receptive at the same time on these inflorescences.

A different mechanism of butterfly-wing pollination occurs in species with 'bowl-brush' flowers, where the anthers remain below or near the edge of the tepals. Bowl-brush flowers are generally presented as a single flower or in a raceme. *Clivia miniata* (which has a round umbellate inflorescence but without overlapping flowers, Fig. 2C), *Cyrtanthus elatus* (Fig. 2B), *Cyrtanthus flammosus*, *Cyrtanthus guthrieae* and *Cyrtanthus taitii* all conform to the bowl-brush floral type. The bowl-brush morphology means that pollination occurs within one flower in a single visit, whereas the open-brush morphology allows pollination to occur to multiple flowers in an inflorescence during a single visit.

Johnson & Bond (1994) described a type of butterfly behaviour they called 'inspection visits', involving the contact of flowers by a butterfly without any feeding attempt made. This behaviour was seen in *Clivia miniata*, and pollen was found to be deposited when butterflies brushed past the flowers or ventured deeper into the bowl flower to feed (Kiepiel & Johnson, 2014). An open-brush morphology effectively utilizes this behaviour, as a butterfly during an inspection visit could easily contact the strongly exserted anthers and stigmas and result in the pollination of several flowers at once, even without necessarily feeding. Such behaviour of butterflies toward flowers was observed for *S. multiflorus*.

The high level of self-incompatibility observed for *S. multiflorus* subsp. *katherinae* implies a near-complete reliance on pollinators for cross-pollination and seed production. The pattern of pollinator activity and plant fecundity found in this study are consistent with the vulnerability of *S. multiflorus* subsp. *katherinae* to disruption of pollinator populations. In the fragmented population of BN, which is in a more urban environment, an absence of butterflies was associated with a ten-fold reduction in fruit set, whereas at the more natural and less disturbed population at VC, high levels of pollinator activity were associated with successful pollination and fruit set. Similar consequences of habitat fragmentation were found for another amaryllid, *Brunsvigia radulosa* Herb., although this species is not pollinated via butterfly wings (Ward & Johnson, 2005). PTE for *S. multiflorus* subsp. *katherinae* was 1.47% at VC in 2018. Although this provides insight into pollen transfer, the calculated value may represent only a small fraction of the possible variation in PTE in this population over time (Peter & Johnson, 2009). Furthermore, PTE varies enormously between species and this may in part represent limitations of the stigmatic surface area (Gong & Huang, 2014). The small stigmas of *S. multiflorus* contact only a tiny fraction of the wing surface of butterflies, suggesting that the advantage of butterfly-wing pollination may lie not in PTE per se, but rather in the increase in pollen carryover from flower to flower, which would drastically reduce the incidence of geitonogamous self-pollination. Curiously, the single visits by butterflies resulted in a higher number of pollen grains on stigmas than in our survey of old stigmas. This could be due to our immediate inspection of flowers for the single visit experiments, whereas pollen grains on older flowers that were not anchored by pollen tubes may have eventually fallen off the stigmas as they aged.

Several other *Scadoxus* spp., which occur outside South Africa, may also be pollinated via butterfly wings, given their similar open-brush floral morphology. *Scadoxus* species we predict to be butterfly-pollinated include *S. cinnabarinus* (Decne.) Friis & Nordal, *S. longifolius* (De Wild. & T.Durand) Friis & Nordal and *S. pole-evansii* (Oberm.) Friis & Nordal. Other *Scadoxus* spp., such as *S. puniceus* (L.) Friis & Nordal, are probably pollinated by birds (Vogel, 2012). To establish whether butterfly-wing pollination in *Scadoxus* is derived from bird pollination, as was shown in the closely related genus *Clivia* (Kiepiel & Johnson, 2014), we would need a better resolved phylogenetic tree for the genus and additional pollinator information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Table S1. Butterfly species diversity present in two populations of *Scadoxus multiflorus* subsp. *katherinae* and in one population of *Scadoxus multiflorus* subsp. *multiflorus*, showing the abundance of butterflies observed to be floral visitors (visitor) to the study species as well as those that did not visit but were present within a 5-m radius of the study plant species (non-visitor).

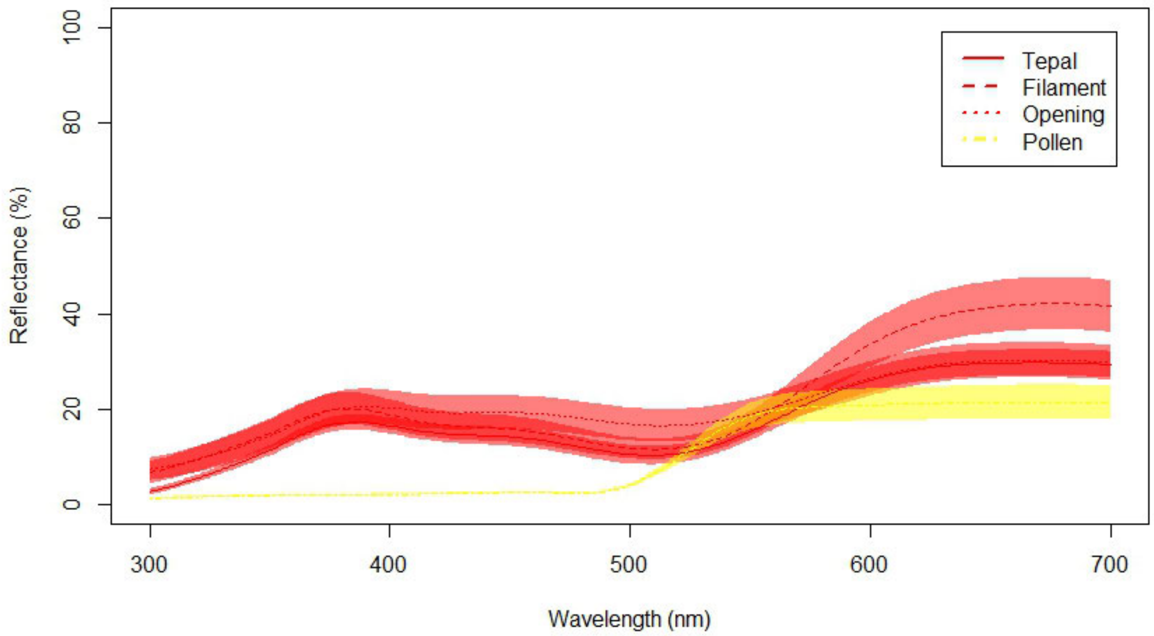
Figure S1. Colour spectra for: A, *Scadoxus multiflorus* subsp. *katherinae*; and B, *Scadoxus multiflorus* subsp. *multiflorus*.

Video S1. Butterfly wing pollination of *Scadoxus multiflorus* subsp. *katherinae*.

Supplemental figures and tables

Supplemental Table 1: Butterfly species present in two populations of *Scadoxus multiflorus* subspecies *katherinae*, showing the number observed in the local habitat and those that visited flowers of the study species.

Place	Family	Species	Common Name	n	Visitor (n)	Wingspan (mm)
VC	Hesperiidae	*Platylesches moritili*	Honey Hopper	7	Visitor (5)	33-35
VC	Lycaenidae	*Pentila tropicalis*	Spotted Buff	6	-	34-44
VC	Nymphalidae	*Bematistes aganice aganice*	Common Wanderer	2	-	70-75
VC		*Amauris niavius dominicanus*	Friar	3	-	78-82
VC		*Eurytela hiarbas angustata*	Pied Piper	9	-	48-55
VC		*Lachnoptera ayresii*	Blotched Leopard	4	-	50-56
VC		*Phalanta phalantha aethiopica*	African Leopard	2	-	43-48
VC		*Junonia tugela tugela*	African Leaf Commodore	7	-	58-64
VC	Papilionidae	*Graphium morania*	White Lady	2	-	55-60
VC		*Graphium policence policenes*	Small Striped Swallowtail	2	-	60-65
VC		*Graphium porthaon porthaon*	Cream-Striped Swordtail	2	-	60-65
VC		*Papilio ophidicephalus*	Emporer Swallowtail	6	Visitor (3)	100-150
VC		*Papilio dardanus cenea*	Mocker Swallowtail	111	Visitor (28)	90-110
VC		*Papilio nireus lyaeus*	Green-banded Swallowtail	15	-	85-95
VC	Pieridae	*Eurema brigitta brigitta*	Broad-bordered Grass Yellow	1	-	30-35
VC		*Nephronia argia*	Large Vagrant	6	Visitor (2)	48-70
BN	Nymphalidae	*Hypolimnas deceptor deceptor*	Deceptive Diadem	3	-	70-80



Supplemental Figure 1: Colour spectra for *Scadoxus multiflorus* subspecies *katherinae*.

Supplemental video 1. Butterfly wing pollination of *Scadoxus multiflorus* subsp. *katherinae*.

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CHAPTER 3: FOR THE BIRDS? CONTRASTING POLLINATION AND BREEDING SYSTEMS OF THE PAINTBRUSH LILIES *SCADOXUS PUNICEUS* AND *S. MEMBRANACEUS* (AMARYLLIDACEAE)

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ORIGINAL ARTICLE

For the birds? Contrasting pollination and breeding systems of the paintbrush lilies *Scadoxus puniceus* and *S. membranaceus* (Amaryllidaceae)

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Abstract

Adaptation to pollinators can include the spatial arrangement of flowers on inflorescences. A brush-like arrangement of flowers has evolved in multiple plant families. The accessible rewards in these "brush flowers" are often utilized by both birds and insects, making it challenging to assess which are the main contributors to pollination. We investigated two such species, *Scadoxus puniceus* and *S. membranaceus*, by means of camera trapping, direct observations, and selective exclusion and controlled pollination experiments in order to determine the extent to which sunbirds contribute to their pollination. We found that flowers of *S. puniceus* are frequently visited by sunbirds, butterflies and honeybees, whereas those of *S. membranaceus* are rarely visited. Birds typically perch directly on the densely packed flowers of *S. puniceus* and pick up large, visible pollen loads on their beaks, heads and feet as they feed on nectar. The flowers of this species match the bills of the sunbirds in length, and the floral nectar properties are also consistent with specialisation for pollination by sunbirds. Butterflies feed on the nectar of *S. puniceus*, but seldom contact the reproductive structures, likely limiting their effectiveness for pollen transfer. Selective exclusion of larger visitors, including birds and butterflies, negatively affected fruit and seed set for both species. Controlled pollination experiments revealed that *S. puniceus* is self-incompatible, whereas *S. membranaceus* is self-compatible and can set fruit autonomously. This study reveals that these two paintbrush lilies which are considered to be closely related have divergent pollination and breeding systems despite sharing similar inflorescence architecture.

Keywords Breeding system · Floral adaptation · Inflorescence architecture · Selective exclusion · Sunbird pollination

Introduction

Birds are important pollinators in almost all of the world's ecosystems (Krauss et al. 2017; Pauw 2019). Flowers adapted for bird pollination are highly variable in shape, colour and their inflorescence arrangement (Pauw 2019). Whereas most bird-pollinated flowers are tubular, a significant proportion lack a distinct floral tube and are densely packed into brush-like inflorescences (Ford et al. 1979). This form of inflorescence architecture has evolved in bird-pollinated members of several plant families, including Proteaceae and Myrtaceae (Collins and Rebelo 1987; Beardsell et al. 1993). These "brush flowers" often pose a conundrum as they show evidence of being specialised for bird pollination in traits such as bright red or orange colours, lack of scent and large nectar volumes, yet are also heavily utilised and visited by insects because of the copious amounts of exposed pollen. For such species, it has proved difficult to clearly identify the roles of birds versus insects as pollinators (Collins and Rebelo 1987; Steenhuisen et al. 2012). One approach has been to use selective exclusion of birds through caging experiments (Wright and Giliomee 1991; Ratto et al. 2018). Caging experiments with *Metrosideros excelsa* Sol. ex Gaertn., for example, revealed this species with brush flowers to be primarily pollinated by birds, despite Myrtaceae being predominantly pollinated by bees (Beardsell et al. 1993; Schmidt-Adam et al. 2009). Caging experiments involving some Proteaceae species with brush flowers have shown insects to be effective pollinators (Gilbin et al. 2017); while others have identified birds as the

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primary pollinators (Vaughton 1996). Species with brush flowers are found in several genera in the Amaryllidaceae, but their reproductive biology has not been previously studied (Duncan et al. 2017).

In this study, we examined aspects of the reproductive biology of *Scadoxus puniceus* (L.) Friis & Nordal and *S. membranaceus* (Baker) Friis & Nordal. These species have been considered closely related on morphological grounds (Nordal and Duncan 1984), and this has recently been confirmed using molecular data (Charlotte Bjorå, University of Oslo, in preparation). The genus *Scadoxus* Raf. exhibits a wide variation in inflorescence architecture (Duncan 2001) and was recognised as a separate evolutionary line from *Haemanthus* L. by Friis and Nordal (1976). A recent study of the fireball lily *Scadoxus multiflorus* Raf., which has flowers arranged in a lax ball-shaped umbel, revealed a specialised pollination system, whereby pollen was transferred via butterfly wings (Butler and Johnson 2020). The flower and inflorescence structure of *S. multiflorus* differs markedly from that of *S. puniceus* and *S. membranaceus*, which have compact brush-like inflorescences (Fig. 1a, b) (Duncan et al. 2017), leading to our hypothesis that the latter two species have evolved a different pollination strategy. Vogel (1954) assigned floral syndromes for South African plant species based on their floral characteristics and categorized *S. puniceus* as ornithophilous (bird-adapted) (Vogel 1954, 2012). Given the similarity of their brush-like inflorescence structure to those of many Proteaceae and Myrtaceae, we hypothesized that *S. puniceus* and *S. membranaceus* would be pollinated primarily by sunbirds.

To determine the extent to which birds contribute to the pollination of *Scadoxus puniceus* and *S. membranaceus*, we investigated (1) the floral biology (including floral dimensions and nectar properties); (2) the breeding system, including the degree of self-compatibility and dependence on pollinators for reproductive success; (3) the identity, frequency and behaviour of floral visitors; and (4) the importance of bird visitation for overall fruit and seed set.

Materials and methods

Study species and sites

Scadoxus puniceus occurs in forest patches and bush clumps in south-eastern Africa and flowers primarily in September (Pooley 1998). We studied this species at seven sites: Vernon Crookes Nature Reserve (30° 15′ 48″ S, 30° 36′ 37″ E; henceforth, VC) where the plants grow in soils covered by leaf litter in rocky habitat within bush clumps surrounded by grassland; Entumeni Nature Reserve (28° 52′ 34″ S, 31° 22′ 09″ E; EN) which was a fully forested habitat; three sites in forest clearing habitat in the Karkloof, which were all 2 km apart, Shawswood stream (29° 18′ 30″ S, 30° 18′ 33″ E; SS), Karkloof forest (29° 18′ 40″ S, 30° 17′ 02″ E; KF), and Mares Tail Falls at Mount Gilboa Nature Reserve (29° 17′ 41″ S, 30° 17′ 58″ E; MF); the botanical gardens of the Pietermaritzburg campus of the University of Kwa-Zulu Natal (29° 37′ 27″ S, 30° 24′ 15″ E; BG) and a private garden just outside of Pietermaritzburg (29°47′32.8″S 30° 28′ 02.1″ E; PG).

Scadoxus membranaceus (Baker) Friis & Nordal occurs in dune forest habitats along the eastern coast of South Africa and flowers primarily in February (Pooley 1998). We studied this species at three sites: Oribi Gorge Nature Reserve (30° 40′ 57″ S, 30° 18′ 42″ E; henceforth, OG) where plants grow in soils covered by leaf litter in rocky habitat in a forested gorge, and the coastal dune forest adjacent to the Beachwood Golf Course of the Durban Country Club (29° 47′ 07″ S, 31°03′ 03″ E; henceforth, DC). Direct observations were also done in the coastal dune forest adjacent to the Scottburgh golf course (30° 17′ 33.9″ S, 30° 45′ 17.7″ E; SB).

The flowers of both species are orange-red and compactly packed in a brush-like inflorescence (hence the common name, “paintbrush lily”). The umbel of *S. puniceus* varies from 50 to 150 mm in width on a peduncle which is 120–750 mm in length, while the umbel of *S. membranaceus* varies from 40 to 90 mm in width on a peduncle which is 140–250 mm in length (Duncan et al. 2017). Each flower produces three ovules and develops into large fleshy berries (Duncan et al. 2017). Voucher specimens for *S. puniceus* (Butler 93) and *S. membranaceus* (Butler 90) were deposited in the BEWS herbarium (NU) at the University of Kwa-Zulu Natal, Pietermaritzburg.

Floral biology

We measured, using a steel ruler, the dimensions of floral parts relevant for pollination: stamen exsertion (the distance from the mouth of the floral tube to the tip of the anthers); style exsertion (the extent to which the style protruded from the tube); herkogamy (the distance between the stigma and its closest anther); and floral tube length. Nectar volume was measured using 5 µl glass micropipettes (Blaubrand, Darmstadt, Germany), and sugar concentration was determined using a handheld refractometer (Bellingham & Stanley, Hampshire, the UK). These nectar measurements were conducted in the middle of the day using flowers from inflorescences that were open to visitation. We measured the standing nectar crop to directly measure the quantity available to visitors. We also counted the number of flowers per inflorescence for several plants for both species. Floral scent was not apparent to the human nose and was not analysed further. Other studies have found that scent is not important in bird pollination systems (Knudsen et al. 2004; Van der Niet et al. 2014).

Controlled pollination

To establish whether the species are genetically self-incompatible and to quantify the extent of reliance on pollinators for reproduction, we carried out controlled hand pollinations. The breeding system experiments were conducted at BG for *S. puniceus* and at DC for *S. membranaceus*. Each treatment was applied to individual inflorescences, as the compact packing of the flowers did not allow for separate treatments within an inflorescence. The treatments were cross (using pollen from a different individual) or self (using pollen from the same individual) as well as a test for autonomous autogamy, whereby inflorescences were left unmanipulated. Inflorescences were bagged to prevent contamination of pollen by external agents. After four months, once the treated flowers had developed fruits, fruit and seed set were counted.

Visitor observations

Floral visitors were determined using direct observations and with Bushnell Nature View camera traps (model 119740, Bushnell Corporation, Kansas, the USA); for *S. puniceus* during August and September 2018 and 2019 and for *S. membranaceus* during February and April 2018 and 2019. Visitors were observed using direct observations for a total of 31 h over 12 days for *S. puniceus*, and 39 h over 13 days for *S. membranceus*. (Details of visitor observations and camera trap days are provided in Table 2.) For *S. puniceus*, visitors were observed directly at every site except for EN and SS, typically for 2–3 h a day around mid-day when both butterflies and birds were active (Fig. 2). For *S. membranaceus*, visitors were observed directly on four days at an additional site, SB, in 2018. Camera traps monitored visitation over two flowering seasons: between two and six cameras were set up for eight of the ten sites for a total of 103 camera trap days for *S. puniceus*, and seven cameras for a total of 102 camera trap days for *S. membranaceus*. Cameras were set at distances of 600 mm from inflorescences, apart from a few cases for *S. puniceus* where cameras were set at 1000 mm (five out of a total of 24) to increase the field of coverage. The probability that Bushnell 119740 cameras will record birds at a distance of 600 mm is c. 75% (Ortmann and Johnson 2020). The cameras were set up in 24 h mode at maximum sensitivity, with infrared illumination enabled and a high shutter speed, taking both still photographs and videos of up to a minute in length. We reviewed the video footage and determined the identity of the visitor species and recorded their activity in relation to the flowers, including bout length (time elapsed from first contact with a flower until departure); the body part in contact with the floral reproductive parts; and overall visitor frequency. We captured representative visitor individuals where possible and counted the number of pollen grains on various body parts. We also examined pollen on two honeybees (*Apis mellifera*) caught after visiting *S. puniceus* at VC, as well as two honeybees and a female double-collared sunbird (*Cinnyris afer*) caught after visiting *S. puniceus* at PG.

Selective exclusion

To assess the importance of different visitors as pollinators, we used cages (mesh diameter = 12.0 × 16.5 mm) which excluded larger visitors such as birds but allowed access to smaller visitors such as honeybees. Cages were placed when the inflorescences were at the bud stage. Once the treated flowers had developed fruits, fruit and seed set were counted. For *S. puniceus*, six inflorescences were caged, and nine inflorescences were left as controls to set fruit naturally at VC and a further eight inflorescences were caged, and eight control inflorescences were left to set fruit naturally at SF. We also collected natural fecundity data for a further 26 *S. puniceus* plants at VC and nine from EN in 2019. For *S. membranaceus*, 12 inflorescences were caged, and four inflorescences were left to set fruit naturally at DC.

Statistical analyses

Trait measurements were compared between the two species using independent samples t-tests. All other analyses involved generalised linear models (GLM's) in SPSS version 26 (IBM Corp.). The duration of foraging bouts was log-transformed and modelled with a gaussian distribution and an identity link function. The number of probes per foraging bout was analysed with a negative binomial distribution and log link function. The proportions of flowers that set fruit and number of seeds per fruit in each treatment were compared using a binomial distribution and logit link function and a negative binomial distribution and log link function, respectively. The natural log of the number of fruits per inflorescence was used as an offset to convert seeds per inflorescence to a rate of seeds per fruit. For the breeding system experiment, treatment group means were compared using the sequential Šidák method. For the analysis of the effects of caging of *S. puniceus*, site and the interaction of site and treatment were included as fixed factors in the model. For statistical comparison of natural levels of fecundity among species, we nested year within site within species for the model structure. For graphical representation of mean proportions and standard errors, data were back-transformed from the logit or log scales. An index of self-incompatibility was calculated for each species as 1 – the proportion of seeds per flower set by the self-pollination treatment / the proportion of seeds set per flower by the cross-pollination treatment, where a value of greater than 0.8 is an indicator of self-incompatibility (Lloyd 1965; Raduski et al. 2012). An index of autonomous self-pollination (IAS) was calculated as the proportion of seeds set per flower of plants bagged with no treatment / the proportion of seeds set per flower by the cross-pollination treatment (Lloyd and Schoen 1992).

Results

Floral traits

Scadoxus membranaceus exhibited longer styles and stamens than *S. puniceus*, resulting in a higher degree of herkogamy as well as overall flower depth (Table 1). There was, however, no significant difference between the two species in terms of tube length, nectar volume and sucrose concentration (Table 1). The overall flower depth (tube length + stamen length) (Table 1) closely matches the tongue lengths of the sunbirds found in southern Africa, which vary from 25 to 40 mm, as described by Downs (2004). The number of flowers per inflorescence varied significantly, with *S. membranaceus* having fewer than *S. puniceus* (Table 1).

Controlled pollination

For *Scadoxus puniceus*, the number of fruits per flower and seeds per fruit were significantly lower for flowers assigned to self and autogamy treatments than for flowers subjected to the cross-pollination treatment (Fig. 3a, b). For *S. membranaceus,* no significant differences were recorded in terms of fruits per flower or seeds per fruit (Fig. 3c, d). An ISI value of 0.85 was recorded for *S. puniceus*, and an ISI value of 0.08 for *S. membranaceus*, indicating that the former species is self-incompatible, while the latter is self-compatible. An IAS of 0.07 was recorded for *S. puniceus* and a value of 0.79 for *S. membranaceus*, indicating that the latter species is capable of autonomous self-pollination, whereas the former species is not.

Visitor observations

The primary visitors of *Scadoxus puniceus* recorded on video were sunbirds ($n = 191$; Fig. 1c–f; Online Resource 1), butterflies ($n = 31$; Fig. 1 h; Online Resource 2), and generalist birds ($n = 5$; Table 2; Fig. 1). We also directly observed c. 95 foraging bouts by sunbirds and 62 foraging bouts by butterflies while working in the study populations. Most of the butterflies were observed in forest habitat at the Karkloof sites (KF, MF, SS; Table 2) and were secondary to sunbirds in terms of visitation frequency at two of these sites, though not at MF (Table 2). Honeybees were rarely recorded on video on account of their small size, but hundreds of individuals were observed collecting pollen from *S. puniceus* flowers during the course of the study (Fig. 1b, g). Bees landed directly on the anthers in order to collect pollen and did not display nectar feeding behaviour.

The camera traps revealed that the activity of sunbirds peaked at mid-morning before dropping during the afternoon and rising again slightly in the early evening (Fig. 2). Similar patterns of activity were noted for butterflies, but the numbers of generalist birds were too low to characterise activity patterns (Fig. 2). Of the total number of foraging bouts caught on camera and through direct observations in which visitors contacted the reproductive parts of flowers, 84% involved sunbirds, 2% involved generalist birds, and 14% involved butterflies (Table 3). Foraging by *Papilio ophidicephalus*, which was the most common butterfly visitor, frequently involved settling on the inflorescence to feed, such that the feet, proboscis and head contacted flowers (Fig. 2; Table 2; Online Resource 2). *Nepheronia argia* butterflies, the second most common butterfly visitor, more frequently settled on the spathe of the inflorescence and so fed from the side, resulting in no floral contact with their feet or heads (Online Resource 2). Of butterfly foraging bouts for which this detail was visible, approximately two-thirds included at least one instance of contact with the head with reproductive parts, but only 6% involved contact of butterfly wings with anthers or stigmas (Table 2). Of the total foraging bouts by sunbirds, 78% involved contact with the

Table 1 Floral trait measurements for *Scadoxus puniceus* and *S. membranaceus*

Measurement	*Scadoxus puniceus*	*Scadoxus membranaceus*	*t*	*df*	*p*
Number of flowers per inflorescence	106.53 ± 8.90 (34)	37.43 ± 5.46 (7)	3.46	39	0.001
Stamen exsertion (mm)	24.21 ± 0.38 (150, 30)	33.29 ± 0.59 (35, 7)	10.06	183	< 0.001
Style exsertion (mm)	24.82 ± 4.88 (150, 30)	33.43 ± 0.69 (35, 7)	9.80	183	< 0.001
Herkogamy (mm)	4.28 ± 0.15 (150, 30)	6.29 ± 0.53 (37, 5)	4.64	183	< 0.001
Tube length (mm)	6.85 ± 0.15 (150, 30)	7.00 ± 0.26 (37, 5)	0.06	183	0.96
Nectar volume (µl)	3.96 ± 0.54 (75, 15)	5.49 ± 0.88 (25, 5)	1.43	98	0.16
Nectar concentration (%)	15.90 ± 1.48 (75, 15)	17.75 ± 0.75 (25, 5)	0.86	70	0.40

Values are grand means (± SE) calculated across sites, with the number of measured flowers and plants given in parentheses

Fig. 1 Habit and pollinators of *Scadoxus* "paintbrush" lilies (see text for site codes). **a** *Scadoxus membranaceus* at DC, **b** *S. puniceus* at BG, **c** *Nectarinia famosa* at KF, **d** *Cinnyris afer* female at SS, **e** *C. afer* male at SS, **f** *Chalcomitra amethystina* female at BG, **g** *Apis mellifera* at BG, **h** *Papilio demodocus* at SS. All scale bars: 5 cm, except **g** (scale bar: 5 mm). Pollen on visitors is indicated by arrows (**d** and **e**)

flowers' reproductive parts with beaks as well as feet, 18% involved contact with beaks only and 4% feet only (Table 3). There were significant differences among the visitor types in their behaviour on the inflorescences; sunbirds probed the highest number of flowers per plant ($\chi^2 = 17.82$; $p < 0.0001$) and spent the longest time foraging on each plant ($\chi^2 = 10.38$; $p = 0.006$) (Table 3). A single sunbird captured on cultivated plants (PG) had small numbers of *Scadoxus* pollen grains on both the feet (12 pollen grains) and beak (308 pollen grains), but birds photographed in natural populations had very large visible pollen loads on their beaks, probably consisting of hundreds of thousands of pollen grains (Fig. 1d, Online Resource 1). The four captured honeybees carried enormous amounts of pollen in their pollen baskets (> 1 000 000 ± 249901 pollen grains; mean ± SE) and smaller quantities on the rest of their body (305 ± 232 pollen grains).

Only two sunbirds were recorded in camera trap footage of *Scadoxus membranaceus*, both at DC (Table 2) and none were observed standing directly on the flowers. During more than 20 h of direct observations at three sites (DC, OG, and SB) over 12 days, just two honeybee individuals were observed collecting pollen from flowers.

Selective exclusion

For *Scadoxus puniceus*, mean levels of fruit and seeds set per plant in exclusion cages differed significantly from those of open-pollinated plants (Fig. 4a, c). For both fruit and seed set, the GLM analyses revealed a significant difference between sites, VC and SF (fruits per plant: $\chi^2 = 43.26$, $p < 0.0001$; seeds per plant: $\chi^2 = 10.17$, $p = 0.312$). However, the interaction between treatment and site was non-significant in both cases (fruits per plant: $\chi^2 = 0.48$, $p = 0.489$; seeds per plant: $\chi^2 = 1.02$, $p = 0.312$). For seeds per fruit, no significant difference was found between caged and natural treatments (Fig. 4 b) as well as for site ($\chi^2 = 0.37$, $p = 0.544$), and the interaction was also non-significant ($\chi^2 = 0.024$, $p = 0.878$). There was a difference in the mean number of flowers per inflorescence between the two study sites (VC = 198 flowers; SF = 87 flowers). In the case of *S. membranaceus*, caging resulted in a significant reduction in fruits and seeds per plant (Fig. 4d, f), but this was not the case for seeds per fruit (Fig. 4e).

Overall, the fecundity of naturally pollinated plants did not differ significantly between *S. puniceus* and *S.*

Table 2 Numbers of floral visitors to *Scadoxus puniceus* and *S. membranaceus* captured on camera traps and through direct observation at ten study sites (see methods for site details). Bold type indicates totals for each pollinator functional group

	S. puniceus											
Study site	BG		EN	KF		MF		SS	VC		PG	Total
Observation method	Camera	Direct	Camera	Camera	Direct	Camera	Direct	Camera	Camera	Direct	Direct	
Days	10	3	1	9	1	9	1	14	60	6	1	
No. cameras/observers	2	1	6	4	1	4	1	2	10	1	2	
Sunbirds												**300**
Chalcomitra amethystina	8	–	–	–	60	5	1	5	56	–	2	139
Cinnyris afer	–	2	–	1	20	10	3	13	70	–	6	125
Cinnyris talatala	2	–	–									2
Cyanomitra olivacea	–	–	20	–	–	–	–	–	–	–	–	20
Nectarinia famosa	–	–	–	1	12	–	1	–	–	–	–	14
Generalist birds												**6**
Ploceus capensis	–	–	–	–	–	–	–	–	1	–	–	1
Zosterops capensis	4	–	–	–	–	–	–	–	1	–	–	5
Butterflies												**93**
Coeliades keithloa keithloa	–	–	1	–	–	–	–	–	–	–	–	1
Nepheronia argia	–	–	–	–	–	4	18	–	1	–	–	23
Papilio ophidicephalus	–	–	–	6	4	10	40	9	–	–	–	69

	S. membranaceus					
Study site	DC		OG		SB	Total
Observation method	Camera	Direct	Camera	Direct	Direct	
Days	38	6	64	4	4	
No. cameras/observers	5	3	2	1	3	
Sunbirds						
Cyanomitra olivacea	2	–	–	–	–	2

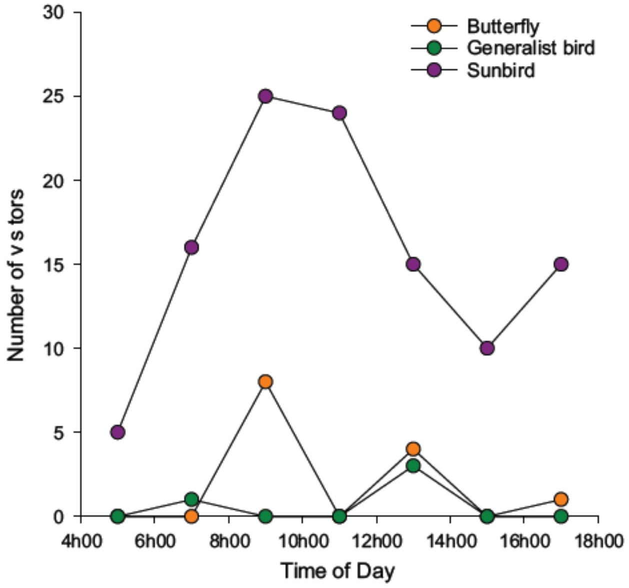

Fig. 2 Diel patterns of floral visitation in populations of *Scadoxus puniceus* from camera trap footage

membranaceus (fruits per plant: *S. puniceus* $= 61.73 \pm 7.33$; *S. membranaceus* $= 29 \pm 8.43$, $\chi^2 = 1.87$, $p = 0.177$; seeds per plant: *S. puniceus* $= 78 \pm 11.74$, *S. membranaceus* $= 35.5 \pm 12.04$, $\chi^2 = 3.72$, $p = 0.06$).

Discussion

Although *Scadoxus puniceus* and *S. membranaceus* are similar in their inflorescence architecture and floral rewards (Table 1), flowers of the former are visited frequently by birds and insects including bees and butterflies, while those of the latter are seemingly rarely visited (Table 2). Breeding experiments revealed that *S. puniceus* is self-incompatible, whereas *S. membranaceus* is largely self-compatible (Fig. 3). Flower dimensions, flower colour, lack of scent, and nectar properties of *S. puniceus* are largely consistent with pollination by sunbirds (Table 1). The mean nectar volume per flower for both species (Table 3) is lower than that expected for sunbird-pollinated flowers (10–30 µl). However, this may reflect sampling of standing nectar rather than

Table 3 Characteristics of foraging behaviour of birds and butterflies feeding on inflorescences of *Scadoxus puniceus* and *S. membranaceus* as caught on camera trap footage. Percent of foraging bouts that include contact with reproductive parts of flowers, mean number of probes and mean duration of foraging bouts

Species	Visitor	*n*	Body part contacting anthers or stigma (% of foraging bouts)							Number of probes	Duration of foraging bout (s)
			Bird body parts			Butterfly body parts					
			Beak	Feet	Beak + Feet	Proboscis only	Proboscis + head	Proboscis + body	Wings		
S. puniceus	Sunbird	191	18	4	78	0				41.04 ± 2.95^{A}	95.35 ± 9.00^{A}
	Generalist bird	5	80	0	20	0				9.40 ± 4.47^{B}	35.80 ± 9.61^{B}
	Butterfly	31	0	0	0	32	65	3	4	18.29 ± 2.55^{B}	29.10 ± 5.11^{B}
S. membranaceus	Sunbird	2	100	0	0	0				9	30

Means that share superscript letters within a column are not significantly different

absolute nectar production. Furthermore, inflorescences have up to 150 flowers, so the amount of nectar produced per inflorescence is substantial. The mean nectar concentration (Table 3) in the two species is within the range expected for sunbird-pollinated flowers (15–25%) (Table 1) (Johnson and Nicolson 2008). Our evidence for bird pollination of *S. puniceus* is based primarily on the observation that birds were frequent visitors, made obvious contact with the reproductive parts, and carried large pollen loads (Fig. 1 c-f; Table 2; Online Resource 1). Interestingly, the sunbirds carried pollen on their beaks and heads as well as their feet when they perched atop the inflorescence (Table 3). Pollen transfer via bird feet has been recorded in a number of different plant species (Frost and Frost 1981; Westerkamp 1990; Johnson and Brown 2004; Coombs and Peter 2009).

Results of camera trapping and direct observations showed that although butterflies are not uncommon visitors to *S. puniceus* (Table 2), they seldom contact floral reproductive structures (Table 3, Online Resource 2) and are therefore unlikely to play a major role in pollen transfer in this species. Butterflies were occasionally visitors at three forest sites in the Karkloof and were the most frequent visitor at one of these sites (MF) (Table 2). At six of the seven study sites, butterflies were secondary to sunbird visitors in terms of both abundance and species diversity (Table 2). The behaviour of the majority of these butterfly visitors also differed from the sunbirds by having shorter bout lengths and fewer number of probes per bout (Table 3). Together these results suggest that butterflies are less effective than sunbirds as pollinators of *S. puniceus*. Thus, the overall reductions in fruit and seed set when birds and butterflies were excluded from inflorescences (Fig. 4), in combination with the predominance of birds at most of the studied populations, indicate that birds are likely the primary pollinators of both *Scadoxus* species.

Honeybees were also frequent visitors to *S. puniceus* flowers and the exsertion of the stamens and stigmas allows for potential pollination by visiting pollen-collecting honeybees (Fig. 1 g). It is common for brush inflorescences to attract both insects and nectar-feeding birds and to be pollinated by both groups of animals (Ford et al. 1979; Collins and Rebelo 1987; Beardsell et al. 1993; Vaughton 1996; Gilbin et al. 2017). Honeybees could, however, potentially decrease the amount of pollen available for transfer by sunbirds by acting as pollen thieves (Hargreaves et al. 2012), although it is also clear from the seed set in caged inflorescences of *S. puniceus* (Fig. 4c) that they do play a minor role in pollination. It is generally difficult to identify the actual relative contributions of birds and honeybees to pollination because it is technically difficult to exclude honeybees and not birds (Wright and Giliomee 1991). However, studies such as that of Diller et al. (2019) have quantified the single effectiveness of birds versus bees and found that bees

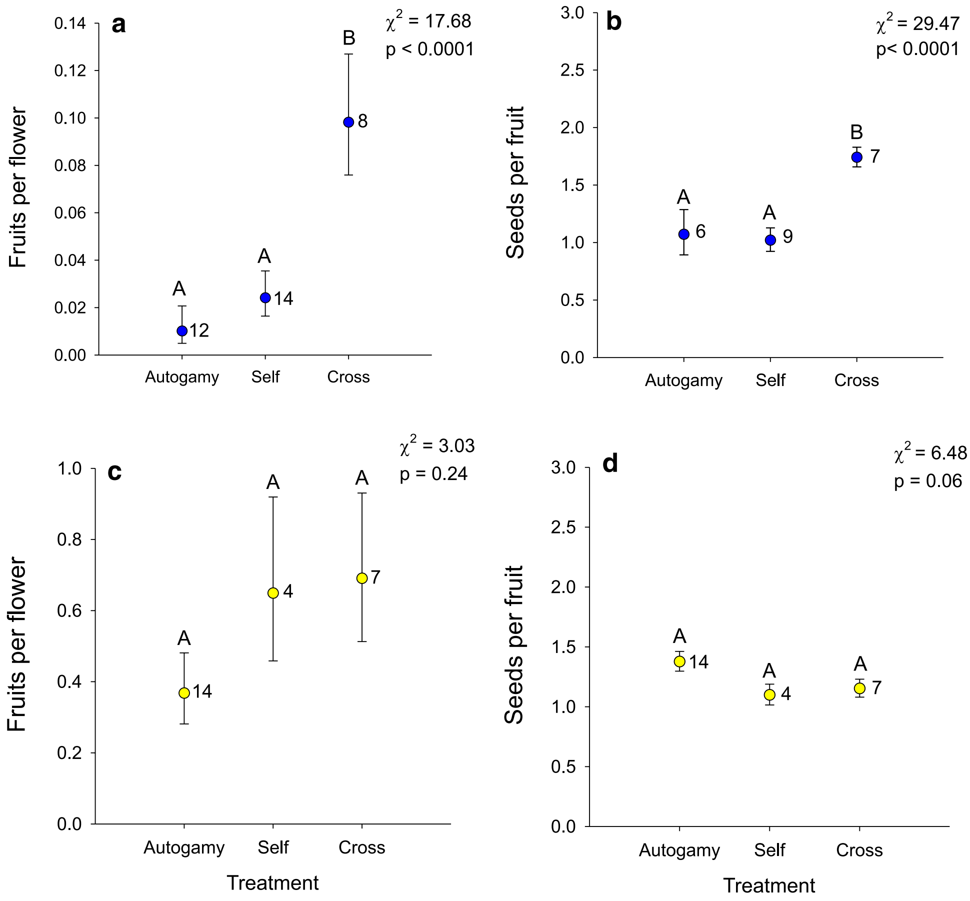

Fig. 3 The effects of controlled hand pollination experiments, with self- or cross-pollen, as well as an unmanipulated treatment to assess the capacity for autogamy, on fecundity of *Scadoxus puniceus* (blue symbols, panels **a** and **b**) and *S. membranaceus* (yellow symbols, **c** and **d**). Symbols indicate mean (± SE) proportion of flowers setting fruit and number of seeds per fruit. Means that share the same letters are not significantly different (Šidák test) and the number of plants sampled is given adjacent to each symbol. For graphical representation of means and standard errors, data were back-transformed from the logit or log scales

can deposit as much pollen as birds, but that the quality of the pollen deposited by bees is often much lower than that deposited by birds.

Our results for *S. membranaceus* show that this species is visited extremely infrequently by sunbirds and honeybees. This low level of visitation is hard to understand since the species produces nectar and has exposed pollen (Table 1; Fig. 1a). However, *S. membranaceus* has significantly fewer flowers per inflorescence than *S. puniceus* (Table 1) which would reduce the amount of available nectar. The species appears to be self-compatible and capable of autonomous seed production (Fig. 3d). Given that caging led to a reduction in fruit and seed set (Fig. 4d, e), we think that the species is not fully autogamous and may therefore benefit to some degree from visits by birds. Evolutionary shifts to self-compatibility and autonomous selfing are thought to be associated with pollinator unpredictably or deficiency (Levin 1972; Kalisz and Vogler 2003; Goodwillie et al. 2005). A study on *Aloe thraskii* Baker, which occurs in a similar dune habitat, showed this species is also self-compatible and has a mixed mating system; a unique feature in the genus as almost all other *Aloe* L. species is self-incompatible (Patrick et al. 2018; Duffy et al. 2020). This was attributed to the species' life history, which involves colonization of shifting dune habitat and isolation from conspecific mating partners during this process (Patrick et al. 2018). Populations of *S. membranaceus* occur en masse in forested dunes close to the sea, often being the only herbaceous plant to cover the forest floor (Fig. 1a). This dune habitat would appear to be pollinator-poor; *Cyanomitra olivacea* was the only species seen to visit *S. membranaceus* and is one of the few sunbird found in this habitat (Newman 2013). A niche shift to occupy this distinct habitat may have resulted in increased self-compatibility to support colonization; much like that suggested for *A. thraskii* (Levin 2010; Patrick et al. 2018; Duffy et al. 2020). Other studies of amaryllids have revealed variation in self-compatibility (Johnson et al. 2019). The genus *Narcissus* L., for example, includes self-compatible species such as *N. longispathus* Pugsley (Barrett et al. 2004), and self-incompatible species such as *N. papyraceus* Ker Gawl. (Simon-Poracr et al. 2015). This suggests that self-incompatibility may be relatively labile in the Amaryllidaceae.

The brush-like inflorescences of *S. puniceus* and *S. membranaceus* (Fig. 1 shows floral architecture of both

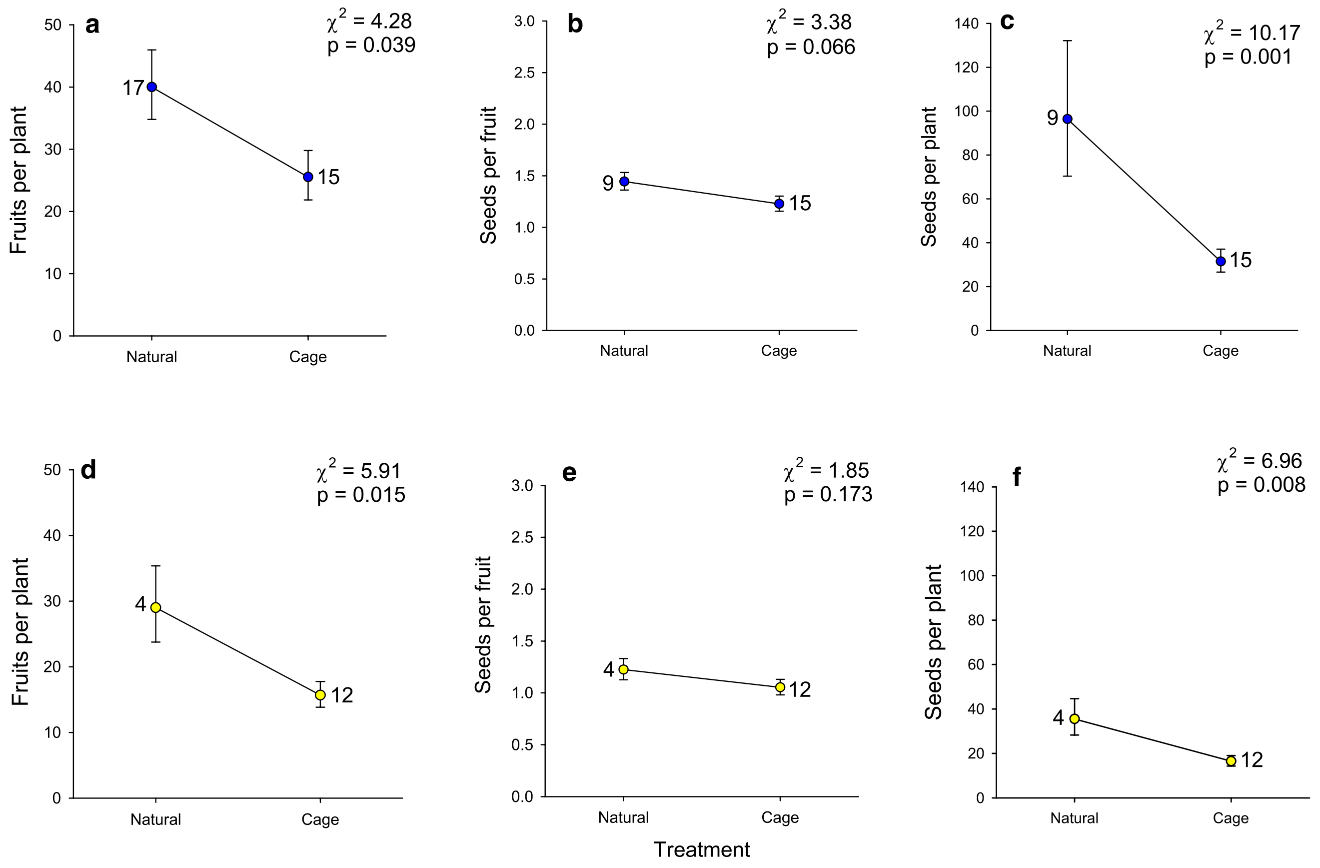

Fig. 4 Effect of caging on fecundity of **a**–**c** *Scadoxus puniceus* and **d**–**f** *S. membranaceus*. Symbols indicate means (± SE), adjacent numbers indicate the number of plants sampled. The statistical results are for the overall treatment effect. Means that share the same letters are not significantly different (Šidák test). For graphical representation of means and standard errors, data were back-transformed from the logit or log scales

species) contrast with the lax ball-shaped inflorescences of the related species *S. multiflorus* which has recently been shown to be specialized for pollination via butterfly wings (Butler and Johnson 2020). Mertens et al. (2020) showed that *S. cinnabarinus* (Decne.) Friss & Nordal displays a similar pollination system to those found by Butler and Johnson (2020) for *S. multiflorus*. The lax arrangement of the flowers of both *S. multiflorus* and *S. cinnabarinus* in a ball-shape means that birds cannot easily access the nectar as the individual flower pedicels do not provide sufficient support (Butler and Johnson 2020; Mertens et al. 2020). Although butterflies do visit *S. puniceus*, very few of them made wing contact with the plants' reproductive parts (Table 3, Online Resource 2), likely due to the compact arrangement of flowers and decreased herkogamy and anther and stigma exsertion. Therefore, although sharing some floral attractive traits, such as red colour and the provision of nectar, inflorescence structure (compact and brush-like vs lax and ball-like) appear to be key for utilisation of different pollination niches by *Scadoxus* species.

The compact brush-like arrangement of flowers in *S. puniceus* can be interpreted as functioning to provide a landing platform for birds. This trait is also found in *S. membranaceus,* despite its apparent reliance mainly on autonomous self-pollination. It is notable that *S. puniceus* and *S. membranaceus* are the only extant species within *Scadoxus* that have a brush-like inflorescence (Duncan et al. 2017). However, *Haemanthus*, the genus considered to be a sister taxon to *Scadoxus*, consists wholly of species with brush-like inflorescences (Snijman 1984). Whether this feature is shared by common descent or evolved independently in the two genera will need to await more detailed phylogenetic analysis.

Information on Electronic Supplementary Material

Online Resource 1. Sunbird visitation to *Scadoxus puniceus*.
Online Resource 2. Butterfly visitation to *Scadoxus puniceus*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00606-021-01798-5.

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Declarations

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Supplemental figures and tables

Supplemental video 1. Sunbird visitation to *Scadoxus puniceus*.

https://drive.google.com/file/d/17bDN9cDX9RHxGbQd9-HOWCfosZAgRHdv/view?usp=sharing

Supplemental video 2. Butterfly visitation to *Scadoxus puniceus*.

https://drive.google.com/file/d/12ngKY-lTmXvF1d0yssTc2WZmP5kilo6k/view?usp=sharing

EVIDENCE FOR SUNBIRD POLLINATION IN AFRICAN BLOOD LILILES: *HAEMANTHUS* (AMARYLLIDACEAE)

Hannah C. Butler & Steven D. Johnson

Abstract

Flowers arranged in ‘paintbrush’ style inflorescences have evolved in several plant families. Their nectar is often accessible to both birds and insects, meaning that observations and experiments are required in order to establish which animal group pollinates these flowers most effectively. The southern African genus *Haemanthus* (Amaryllidaceae) includes more than 20 species, all with paintbrush style inflorescences and flower colour that ranges from white to red. Here we examine the contributions of birds versus insects to the pollination of four *Haemanthus* species varying in flower colour. Direct observations and camera trapping showed that flowers of these *Haemanthus* species are frequently visited by sunbirds as well as by insects. Bird exclusion experiments revealed that *H. coccineus* (red flowers) and *H. deformis* (white flowers) are strongly dependant on sunbirds for pollination, whereas *H. humilis* subsp. *hirsutus* (pink flowers) is co-pollinated by insects. All *Haemanthus* species tested were reliant on pollinators for seed production. Bird pollination appears to be frequent in *Haemanthus* and appears to be correlated with large volumes of nectar in inflorescences, rather than with flower colour or floral and inflorescence morphology.

Keywords: Sunbird pollination – Bird pollination syndrome – Exclusion experiments – Camera trapping – Amaryllidaceae

Introduction

'Paintbrush' style inflorescences have evolved in several plant families, including Proteaceae, Asteraceae, Hyacinthaceae and Amaryllidaceae. The inflorescences are comprised of densely packed flowers and serve as blossoms (Faegri and van der Pijl, 1979). The aggregated flowers collectively provide a perching platform, are not singled out individually by pollinators when foraging, and pollen transfer is generalized and not specific to the flower that is probed. Because flowers in paintbrush style inflorescences have very accessible nectar, they are often visited by a wide range of animals (Beardsell et al., 1993; Butler et al., 2022; Collins and Rebelo, 1987), making it difficult to ascertain if there is any specialization in pollination systems. Although several plant species with paintbrush inflorescences have been shown to be pollinated primarily by birds (Butler et al., 2022; Hingston et al., 2004; Vaughton, 1996), individual flowers with their reduced perianth usually do not conform to the bird pollination syndrome (Faegri and van der Pijl, 1979; Krauss et al., 2017). However, features such as large amounts of nectar per inflorescence (though not necessarily per flower), colour and dimensions nevertheless show convergence with more typical bird-pollinated flowers.

There are several examples of paintbrush style inflorescences that are situated at ground level (geoflory). In *Protea* (Proteaceae) and *Massonia* (Hyacinthaceae) these are strongly associated with pollination by rodents (Biccard and Midgley, 2009; Johnson et al., 2001). Hummingbirds visit the geoflorous blossoms of *Scybalium fungiforme* (Balanophoroaceae) (Amorim et al., 2020). Sunbirds, as compared to hummingbirds, are generally considered to require a perch and in *Babiana ringens*, a modified section of the inflorescence which is situated near ground level provides a perch for sunbirds (Anderson et al., 2005). Geoflory is not, however, a general hinderance to sunbird pollination as several geoflorous South African species, which include *Cytinus sanguineus* (Cytinaceae) (Hobbhahn and Johnson, 2015), *Hyobanche sanguinea* (Orobanchaceae) and *Lachenalia luteola* (Hyacinthaceae) (Turner and Midgley, 2016), are pollinated by sunbirds.

The South African members of the family Amaryllidaceae show an extraordinary range of floral adaptations to various pollinators, ranging from moths, butterflies, long-proboscid flies, bees and other insects, to sunbirds (Balmford et al., 2006; Butler et al., 2022; Butler and Johnson, 2020; Geerts and Pauw, 2012; Johnson et al., 2009; Kiepiel and Johnson, 2014; Manning and Snijman, 2004; Mertens et al., 2020; Newman et al., 2015; Vaughton et al., 2010; Ward and Johnson, 2005). It is likely that multiple pollinator shifts within the family have resulted in speciation. Work on *Clivia*, for example, has shown a shift from bird to butterfly (Kiepiel and Johnson, 2014). Recent research on *Scadoxus puniceus*, an amaryllid with a paintbrush style inflorescence, has shown that the species is adapted to and reliant on sunbird pollination, while the related species *S. multiflorus* with a lax ball-like inflorescence is pollinated by butterflies (Butler et al., 2022; Butler and Johnson, 2020).

The genus *Haemanthus* is the sister taxon to *Scadoxus* and occurs mostly in South Africa, with some species extending into Namibia. Previous authors have suggested that some *Haemanthus* species are pollinated by bees and other insects (Snijman, 1984). Vogel (1954) considered the white flowers of *H. albiflos* Jacq. to conform to the floral syndrome of bee pollination and also reported observations of bees visiting the scented pink flowers of *H. pumilio* Jacq.. Vogel (1954) considered species with red flowers such as *H. rotundifolius* [Ker.-Gawl.] and *H. coccineus* L. to confirm to "ornithophilous" (bird-adapted). Some species with red flowers are visited by butterflies (Johnson and Bond, 1994). However, the morphology of the paintbrush style inflorescence of these species is very similar to those of *Scadoxus puniceus* which has been shown to be bird-pollinated (Butler et al., 2022) which led us to hypothesize that red-flowered *Haemanthus* species are primarily bird-pollinated. Some species, however, such as *H. deformis* Hook.f. and *H. albiflos* have white flowers which is not typical for bird-pollinated plants. *Haemanthus deformis* is geoflorous and occurs in densely wooded habitats, leading us to initially speculate that this particular species may be rodent-pollinated.

We investigated the pollination of four species of *Haemanthus,* two with white flowers (*H. albliflos* and *H. deformis*) one with pinkish flowers (*H. humilis* subspecies *hirsutus* (Baker) Snijman) and one with red flowers (*H. coccineus*). Using camera traps, we determined whether these species are visited by sunbirds. We used exclusion cages to determine the contributions of sunbirds to seed production and full exclusion to establish general reliance on pollinators. We also measured floral traits, such as morphology and nectar properties, to determine their potential match with sunbird pollinators.

Methods

1. Study species and study sites

Haemanthus albiflos Jacq., was studied in 2019 at Oribi Gorge nature reserve (30°42’27.0” S, 30°16’10.1” E). This species occurs in coastal forest belts along the eastern coast of South Africa. Floral measurements were done from a population in the UKZN botanical gardens on the Pietermaritzburg campus (29°37’27” S, 30°24’15” E). *H. deformis* was studied in 2019, 2020, and 2021 at various subsites in Cumberland private nature reserve (29°30’20” S, 30°30’14” E; 29°31’20” S, 30°30’51” E) where it occurs in bush clumps. This species has a very short flower stem such that the inflorescence is on the ground (geoflory). *H. coccineus* was studied in 2021 and 2022 in Hoek-van-die-berg private nature reserve (34°24’50” S, 19°07’27” E). *Haemanthus humilis* subspecies *hirsutus* was studied in 2019 in Moor Park nature reserve (29°04’29” S, 29°48’13” E). This species occurs in rocky outcrops in grassland and savanna vegetation.

2. Floral measurements

A steel ruler was used to make the following measurements for each species: stamen exsertion and style exsertion (the extent to which the stamen or style is exerted from the tube); tube length, and herkogamy. In addition, the amount of nectar was measured using 5µl glass micropipettes (Blaubrand, Darmstadt, Germany). Nectar concentration was measured using a handheld refractometer (Bellingham & Stanley, Hampshire, UK). The nectar measurements were made using standing nectar crop, to best ascertain the amount and concentration of nectar available to visitors. A one-way ANOVA with a Tukey post-hoc test was used to compare the species’ floral measurement.

3. Pollinator observations

Direct observations were as follows: *H. albiflos* for two days (typically 09h00 to 19h00) in the botanical gardens of the university of KwaZulu-Natal (29°37’27” S, 30°24’15” E) and two days in Umtamvuna nature reserve (31°00’20” S 30°09’47.0” E); *H. coccineus* for two days in Hermanus (34°24’39” S 19°16’50” E) and four days in Hoek-van-die-berg private nature reserve; *H. deformis* in Cumberland for seven days; and two days for *H. humilis* subsp. *hirsutus* for two days in Moor Park nature reserve. Bushnell Nature View camera traps (model 1197740, Bushnell corporation, Kansas, USA) were used. These motion trigger cameras were mounted on small tripods and were set at distances of 600 mm from inflorescences. The cameras were set up to record day and night at maximum sensitivity, with infrared illumination enabled and a high shutter speed, taking both still photographs and videos of a minute in length. These settings have been shown to be effective for recording small mammals and birds (Ortmann and Johnson, 2020). We then reviewed the footage to determine animals that visited the inflorescences as well as the number of probes, bout length, and part of body in contact with the reproductive parts for each visitor. For *H. albiflos*, one camera was used for a total of 36 days in 2019. For *H. coccineus*, three were used for a total of nine days in 2022 and two cameras were used for a total of 21 days in 2021. For *H. deformis*, 12 were used in 2020 for 28 days, four were used in 2018 for 26 days, eight were used in 2019 for 21 days, and four were used in 2021 for five days. For *H. humilis* susp. hirsutus, eight were used in 2019 for 15 days.

4. Selection exclusion

Cages (mesh diameter = 12.0 × 16.5 mm) that excluded larger visitors such as birds but allowed smaller visitors such as honeybees, were placed over individual inflorescences at the bud stage. A fully bagged treatment that tested for autonomous autogamy was also performed for *H. deformis* and *H. humilis* subsp. *hirsutus*. For *H. coccineus*, four were caged and nine were controls in 2021. For *H. deformis*, 23 were caged, 10 bagged, and 60 were open controls. For *H. humilis* subsp. *hirsutus*, five were caged, 13 were bagged and 10 were open controls. Resulting fruit and seed set for each treatment was counted. The number of fruits per per plant and number of seeds per plant were statistically compared among treatments using generalized linear models (GLMs) with a negative binomial distribution and log link function, implemented in SPSS 25 (IBM Corp.) Treatment group means were compared using the post-hoc sequential Šidák method.

Results

1. Floral Measurements

The flowers of all *Haemanthus* species investigated were similar in their overall dimensions (Table 1). However, *H. humilis* subsp. *hirsutus* had a relatively small standing crop of nectar per flower compared to the other species which had roughly 20-fold more nectar volume per flower (Table 1). Nectar concentration varied two-fold among the species, with *H. albiflos* having the most dilute nectar and *H. coccineus* having the most concentrated nectar (Table 1).

Table 1. Floral trait measurements for the three *Haemanthus* species investigated. Means that share letters are significantly different.

Haemanthus Species	Stamen exsertion (mm)	Style exsertion (mm)	Tube length (mm)	Nectar amount (µl)	Nectar concentration (%)
H. albiflos	21.06 ± 0.65^{A}	23.11 ± 0.45^{A}	6.13 ± 0.26^{A}	3.79 ± 0.47^{A}	15.56 ± 0.76^{A}
H. coccineus	22.44 ± 0.64^{A}	21.28 ± 0.52^{A}	5.44 ± 0.13^{A}	3.06 ± 0.34^{A}	34.86 ± 2.46^{B}
H. deformis	25.86 ± 0.38^{B}	25.50 ± 0.64^{B}	9.28 ± 0.24^{B}	1.98 ± 0.28^{A}	26.49 ± 1.47^{B}
H. humilis subsp. *hirsutus*	18.93 ± 0.66^{C}	16.21 ± 1.23^{C}	6.50 ± 0.14^{A}	0.11 ± 0.05^{B}	28.60 ± 5.78^{AB}

2. Pollinator observations

Data from motion trigger cameras showed that all species were visited regularly by sunbirds (Table 2, Figure 1; Video S1). *Haemanthus deformis* was visited, in some populations, by bees, which appear to visit the flowers to collect pollen, despite nectar present. The cameras showed that this species is visited extensively by sunbirds (n = 20) which usually perch on the ground when feeding on the flowers due to the very short, almost non-existent, peduncle (Figure 2A and B). This meant that pollen was placed onto the beaks, throats as well as foreheads of the birds (Table 2). Several rodent species (n= 6) were caught on camera in the vicinity of *H. deformis* flowers but never visited (n=87; Table S1). *Micaelamys namaquensis* was the most common at two sites where the flowers occurred, Cato Ridge (29°47’40.99” S; 30°33’58.89” E) and Cumberland (Table S1). Observations of *H. albiflos* showed floral visits by sunbirds which used the longer, more horizontal, peduncles as a perch when feeding. *Haemanthus humilis* subsp. *hirsutus* was visited by sunbirds (n = 16; Figure 2D) and occasionally by long-proboscid flies of the genus *Philoliche* as well as *Amegilla* bees, c. 10 individuals of each. The sunbirds visiting *H. humilis* subsp. *hirsutus* occasionally used the inflorescence as a perch so that their feet contacted the reproductive parts of the flowers (Table 2). *H. coccineus* was also visited by sunbirds (n = 9; Figure 2F), which gripped the peduncle or stood atop the inflorescence, as well as being visited extensively by bees in some populations.

Table 2. Sunbird visitors to three *Haemanthus* species and their mean (± se) number of probes per plant and bout durations and pollen placement. Data based on camera trap footage.

Haemanthus species	Sunbird species	Number of sunbirds	Average number of probes	Average bout length (s)	Contact with floral reproductive structures parts of sunbirds (n = individuals).
H. coccineus	*Cinnyris chalybeus*	2	5.0 ± 2.0	8.0 ± 3.0	Beak (1); Beak + throat (1)
	Nectarinia famosa	7	48.1 ± 10.6	54.3 ± 14.6	Beak (7)
H. deformis	*Chalcomitra amethystina*	7	6.3 ± 1.9	13.6 ± 3.9	Beak + throat (3); Beak + throat + forehead (2)
	Cinnyris talatala	13	13.6 ± 3.1	29.1 ± 8.2	Beak + throat (7); Beak + throat + forehead (3)
H. humilis subsp. *hirsutus*	*Chalcomitra amethystina*	1	22.0	45.0	Beak
	Cinnyris afer	15	23.9 ± 4.3	3.9	Beak (6); Beak + feet (9)

Figure 1. Sunbird visitors to four *Haemanthus* species. (A) *Cinnyris talatala* visiting *H. deformis*. (B) *Chalcomitra amethystina* visiting *H. deformis*. (C) *Cyanomitra* olivacea visiting *H. albiflos*. (D) *Cinnyris afer* visiting *H. humilis* subsp. *hirsutus*. (E) *H. coccineus in situ* in Hermanus. (F) *Cinnyris chalybeus* visiting *H. coccineus*. All scale bars = 25mm, except (E) = 250mm.

3. Caging experiments

When sunbirds were excluded, *H. coccineus* and *H. deformis* produced a lower fruit set than did naturally-pollinated plants (Fig 2ab). For *Haemanthus coccineus*, means of fruit number per naturally-pollinated plant were significantly higher than those of plants in cages, where none of the inflorescences produced any fruit (Figure 2A). For *H. deformis*, means of fruit and seed number per naturally-pollinated plant was significantly higher than those of plants in cages and a bagged treatment resulted in a further reduction in fruit and seed set (Figure 2B). For *H. deformis*, these experiments were done over two years, and there was no significant difference between the years ($\chi^2 = 4.011$; $p = 0.045$). For *H. humilis* subsp. *hirsutus*, means of fruit and seed number per naturally-pollinated plant was not significantly different than those of plants in cages, but a bagged treatment resulted in a significantly lower fruit and seed set (Figure 2CF).

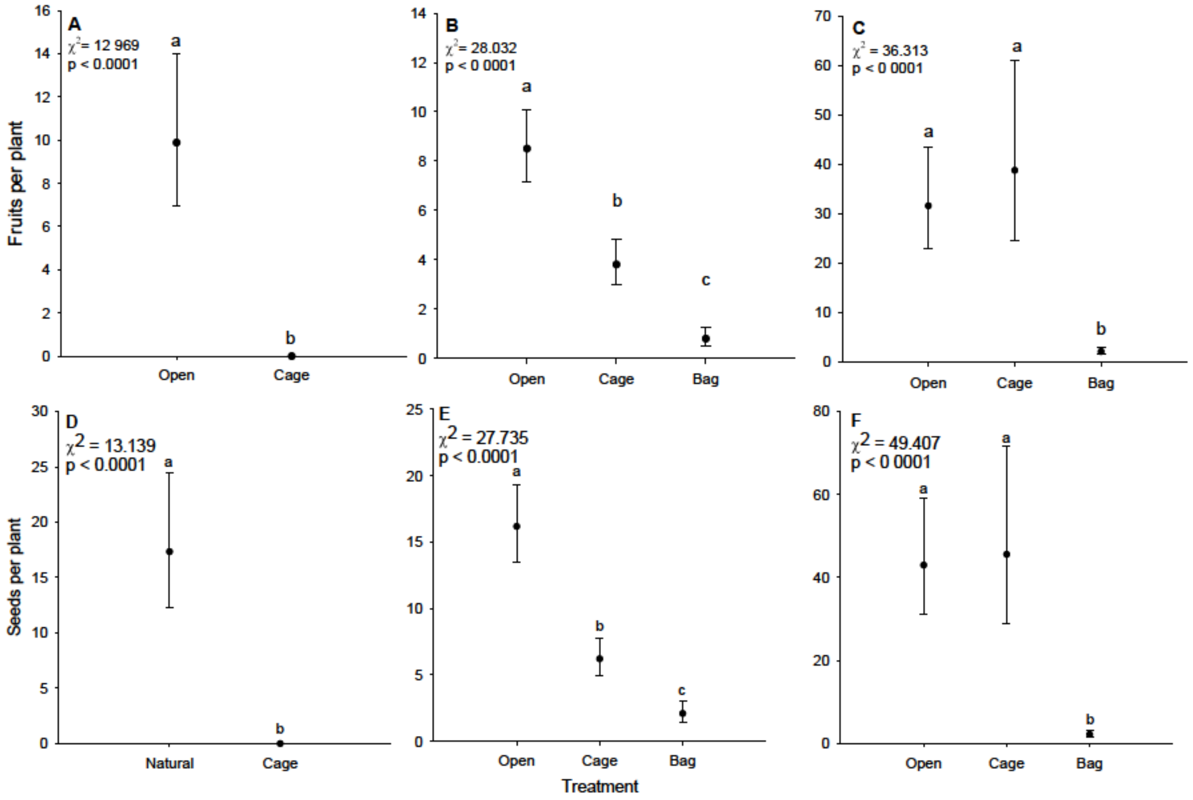

Figure 2. The effect of caging on fecundity in terms of fruit and seed sets of three *Haemanthus* species. (A), (D) – *H. coccineus*; (B), (E) – *H. deformis*; (C), (F) – *H. humilis* subsp. *hirsutus*. Symbols indicate means (± SE), and different letters indicate statistically significant differences. The statistical results are for the overall treatment effect. For graphical representation of means and standard errors, data were back-transformed from the log scale.

4. Discussion

We show here that *Haemanthus deformis* and *H. coccineus* are reliant on sunbird pollination for reproductive success (Figure 1 and 2). This was confirmed through camera trap recording of floral visitors as well as caging experiments which revealed that both species have a very low fruit and seed set when sunbirds are excluded. We also found that *H. albiflos* is visited by sunbirds but did not perform selective exclusions for that species. *H. humilis* subsp. *hirsutus* was visited by both sunbirds and insects such as *Philoliche* flies and *Amegilla* bees. Exclusion of birds did not affect fruit set of this species, indicating that insects contribute strongly to fecundity.

Of particular interest was our finding of exclusive sunbird pollination of the geoflorous inflorescences of *H. deformis*. Rodents were commonly observed in nocturnal footage (Table S1), but did not visit the flowers, indicating that this species is not rodent pollinated, despite the structural similarities of the inflorescences with those of species such as *Massonia depressa* (Johnson et al., 2001) and several *Protea* species (Zoeller et al., 2016). The sunbirds that visit the flowers of *H. deformis* perch on the ground while feeding (Figure 1A and B). Other South African geoflorous species are also sunbird pollinated and include *Hyobanche sanguinea*, *Lachenalia luteola* and *Cytinus sanguineus* (Hobbhahn and Johnson, 2015; Turner and Midgley, 2016). Sunbirds visiting *H. albiflos*, on the other hand, grip the more substantial peduncle of this species whilst feeding (Figure 1C). Sunbirds predominantly used the bracts of *H. coccineus* and *H. humilis* as perches when feeding and sometimes the inflorescence itself (Table 2).

Floral colour is an important signal to pollinators, and can be an indicator of the pollination system (Schiestel and Johnson, 2013). Interestingly, both red- and white-flowered species of *Haemanthus* were found to be bird-pollinated. White-flowered bird-pollinated plats are not common and usually white flowers are correlated with a shift in pollinator away from birds (Cronk and Ojeda, 2008). The white-flowered species *H. deformis* and *H. albiflos* occurs in dense bush clumps or forest and the white colour may render the inflorescences more visible to birds in terms of being conspicuous against the background. *H. humilis* subsp. *hirsutus* has a pale pink colour and we found this species to be co-pollinated by insects. The long-tongued flies of the genus *Philoliche* which visit this species are known to be attracted to different colours, often depending on the food source, but have a particular preference for pale pink flowers (Goldblatt and Manning, 2000; Jersáková et al., 2012). The red-flowered *Haemanthus* species occur in sunny habitats with bright sand, possibly rendering the red inflorescences more conspicuous.

In conclusion, *H. coccineus and H. deformis* and likely also *H. albiflos*, are pollinated by sunbirds. It is likely that sunbird pollination is important for many other *Haemanthus* species. The pink-flowered *H. humilis* subsp. *hirsutus*, however, appears to be co-pollinated by insects and sunbirds. It may be that other pink species (*H. amarylloides*; *H barkerae*; *H tristis*) are more reliant on insect pollinators (such as the endangered *H. pumilio* (Summerfield and van der Walt, 1992; Vogel, 1954)).

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Supplemental material

Video S1. Sunbird visitors to four species of *Haemanthus*.

https://drive.google.com/file/d/1DWpvz8X9EsBKl5xwlLYT902_Zybjg9UF/view?usp=sharing

Table S1. Rodent species caught on camera in the vicinity of *Haemanthus deformis* flowers.

Site	Rodent species	Number of rodents
Cato Ridge	*Grammomys dolichurus*	4
	Micaelamys namaquensis	43
Cumberland	*Grammomys dolichurus*	2
	Lemniscomys rosalia	4
	Micaelamys namaquensis	32
	Suncus infinitesimus	2

CHAPTER 5: SEED DISPERSAL BY MONKEY SPITTING IN *SCADOXUS* (AMARYLLIDACEAE): FRUIT SELECTION, DISPERSAL DISTANCES, AND EFFECTS ON SEED GERMINATION

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Seed dispersal by monkey spitting in *Scadoxus* (Amaryllidaceae): Fruit selection, dispersal distances and effects on seed germination

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Abstract Fleshy fruits are usually associated with ingestion of seeds but can also serve as a reward to animals that discard seeds without ingesting them. We investigated the seed dispersal systems of two South African *Scadoxus* lilies. Like those in some other genera in Amaryllidaceae tribe Haemantheae, seeds of *Scadoxus* are non-orthodox, reputedly poisonous and enclosed within fleshy fruits. The bright red ripe *Scadoxus* fruits attract monkeys, which consume the fleshy fruit and spit out the seeds. Depulping increases the rate of seed germination. Monkeys spit some seeds out in the immediate vicinity of the plant and carry others further away in their cheek pouches (84% of *S. multiflorus* subsp *katherinae* seeds and 78% of *S. puniceus* seeds were dispersed further than 1 m away from the parent plant). Both species occur in very specific spatially restricted habitats; *S. multiflorus* subspecies *katherinae* is confined to patches of swamp within forests, while *S. puniceus* is confined to small bush clumps in a grassland mosaic. Monkey-mediated seed dispersal may be advantageous for these two *Scadoxus* species as it ensures that some seeds are spat out in the immediate spatially restricted habitats of the parent plants, while others are carried through cheek-pouching to more distant habitat patches.

Key words: Amaryllidaceae, monkey seed dispersal, *Scadoxus*, seed distribution, seed germination.

INTRODUCTION

Seed dispersal is an important mechanism for plants to maintain populations and establish new ones (Tiffney 1984; Willson & Traveset 2000). Interactions with seed dispersers play an important role in plant life histories and can determine the distances seeds travel, and the extent to which seeds will germinate and offspring will survive (Tiffney 1984). The development of a fruit layer surrounding the seed was a key driver in the success and diversification of angiosperms and has facilitated the evolution of multiple seed dispersal methods (Herrera 1989; Willson & Traveset 2000). Fleshy fruits usually indicate seed dispersal via zoochory, and their features such as colour and size can influence specificity of associations with groups of animal dispersers (Tiffney 1984; Gautier-Hion *et al.* 1985). Fruit processing by frugivores has an impact on seed dispersal distances and post-dispersal factors such as germination and seedling recruitment (Rowell & Mitchell 1991; Lambert 2002).

Baccate fruits (fleshy fruits that resembles berries) occur in some African members of the Amaryllidaceae belonging to the tribe Haemantheae (Snijman & Linder 1996). Members of the closely related tribe Amaryllideae have dry fruits and are dispersed via various abiotic mechanisms. Baccate fruits seem to have evolved just once in the Amaryllidaceae. (Meerow & Clayton 2004), and Snijman and Meerow (2010) hypothesized that they function to attract animal dispersers. However, the actual mechanisms of dispersal in this clade have been a long-standing mystery as the seeds of Haemantheae are fleshy and recalcitrant (lacking dormancy, also known as 'unorthodox') and would thus not be expected to survive gut passage. A recent study of *Clivia miniata* (Lindl.) Bosse in the Haemantheae by Kiepiel and Johnson (2019) showed that samango monkeys eat the fruits of this species and then spit out the bitter-tasting and probably toxic seeds, thus resulting in their dispersal without being ingested.

Fruits of *Scadoxus* Raf (Amaryllidaceae: Haemantheae) are brightly coloured when ripe and have a succulent pulp. They fit the bird-monkey dispersal syndrome described by Gautier-Hion *et al.* (1985) and are similar in structure to those of *Clivia*. Because of their large size, the lack of a hard seed coat and probable toxicity, we hypothesized that seeds of *Scadoxus* have a dispersal mechanism involving monkey spitting behaviour. Monkey spitting as a form of seed dispersal is also known outside of Amaryllidaceae, and seeds are dispersed either in the immediate vicinity of the mother plant or further away after being held in the monkeys' cheek pouches

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(Lambert 2002). In general, spitting behaviour is expected to result in seed dispersal distances which are much shorter than those arising from ingestion and gut passage, and may thus be of benefit for plants adapted to spatially restricted habitats. In the case of plants with seeds that are recalcitrant (lacking dormancy), dispersal to a particular micro-environment can be critical for seedling establishment (Pammenter & Berjak 2000).

Frugivores can promote seed germination by removing fruit pulp (Rader & Krockenberge 2007). Germination inhibitors can still be present in the pulp of ripe fruits and removal of the endocarp by a disperser can thus play a positive role in seed germination and establishment (Evenari 1949). These factors are particularly relevant for *Scadoxus* given that the seeds are recalcitrant and germination must occur when the seeds still have a high moisture content (Farrant *et al*. 1989). The fruits of *Scadoxus* show a visible colour change from green to red when ripening (Fig. 1a), which may be correlated with seed maturity and germination ability. It may therefore be advantageous to the plant if the disperser showed a preference for the ripest (red) fruits, though, in the case of *C. miniata* monkeys selected green and red fruits equally (Kiepiel & Johnson 2019). We consequently predicted that: (1) *Scadoxus* seeds would be dispersed by monkeys, (2) monkeys would select red over green fruits, (3) the seeds would be spat out close to parent plants and also dispersed longer distances via cheek-pouching, and (4) that both fruit ripeness and fruit pulp removal would have a positive effect on seed germination.

The aims of this study were to determine: (1) whether fruits of *S. multiflorus* subspecies *katherinae* (Baker) Friis & Nordal and *S. puniceus* (L.) Friis & Nordal are dispersed by monkeys, and, if so, to identify the species responsible, (2) to study the fruit-processing behaviour of dispersal agents, including their selection of fruits at different stages of ripeness, (3) to estimate distances that seeds are dispersed and (4) to study the effects of fruit ripeness and fruit pulp removal on the timing and proportion of seed germination.

METHODS

Plant species descriptions

Scadoxus multiflorus subspecies *katherinae* (Baker) Friis & Nordal inhabits swampy areas surrounding streams within coastal forests in eastern Africa. Inflorescences are up to 1.5 m in height and have conspicuous bright red flowers arranged in a lax ball shape, which are pollinated by butterflies (Butler & Johnson 2020). Fruits ripen slowly over 4 months and change from green, through yellow to red in colour. Fruits are up to 20 mm in length and contain up to three large (c. 10 mm diameter), hard, recalcitrant seeds, which are pale cream in colour. Dispersal biology

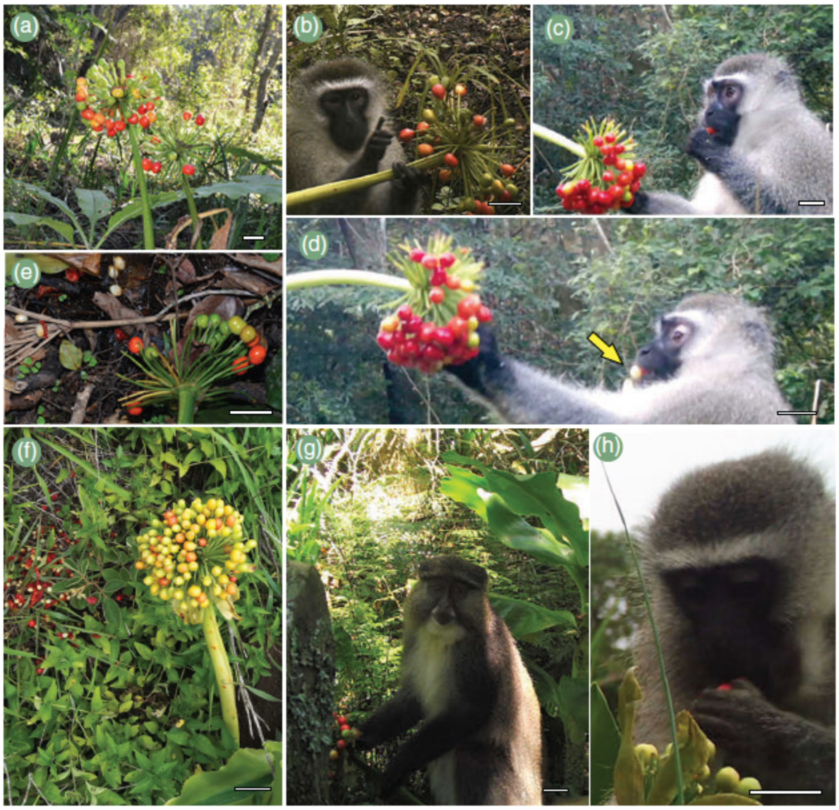

Fig. 1. Animal seed dispersers of *Scadoxus*. (a) *S. multiflorus* subsp. *katherinae* plant with infructescence *in situ*. (b) and (c) Vervet monkeys selecting fruits of *S. multiflorus* subsp. *katherinae*. (d) Vervet monkey handling the fruits of and spitting out seeds and exocarp of *S. multiflorus* subsp. *katherinae* (arrow points to a seed being spat out). (e) An infructescence of *S. multiflorus* subsp. *katherinae* with handled and spat out seeds and exocarps from the plant lying next to it. (f) *S. puniceus* plant with infructescence in situ with handled and spat out seeds and exocarps from the plant lying next to the plant in VC. (g) Samango monkey holding a *S. puniceus* infructescence in KF. (h) Vervet monkey picking fruits from *S. puniceus* in VC. All scale bars = 50 mm.

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of this taxon was studied at the Vernon Crookes Nature Reserve (hereafter referred to as VC; 30°16′17.1” S, 30°35′35.3″ E), in July 2018 and July and August 2019.

Scadoxus puniceus (L.) Friis & Nordal is found within forest patches and bush clumps in eastern southern Africa. Inflorescences are up to 1 m in height, and the flowers, which are tightly packed into a paintbrush shaped inflorescence, are pollinated by birds (Butler *et al.* 2022). The fruits are similar to those of *S. multiflorus* subsp. *katherinae*, although slightly smaller at around 15 mm, with a seed diameter of 10 mm. This study was conducted within Vernon Crookes Nature Reserve (hereafter referred to as VC; 30°15′48” S, 30°36′37″ E0), in November, December and January 2018, and November in 2019, as well as in the forests of the Shawswood property in the Karkloof (hereafter referred to as KF; 29°18′40” S, 30°17′02″ E), in December and January 2019.

Animal observations and behaviour

To determine the identity and behaviour of animal seed dispersers, we used Bushnell Nature View camera traps (models 119 740 and 119 874, Bushnell Corporation, Kansas, USA). The cameras were set up for 24 h to take photographs (three per motion trigger) as well as videos for a length of 30 s. Each camera was focused on a single infructescence. For *S. multiflorus* subsp. *katherinae*, 11 cameras were used for 21 days in 2018 and six cameras for 18 days in 2019. For *S. puniceus* at VC, four cameras were used for 19 days in 2018 and three cameras for 7 days in 2019, and at KF, five cameras were used for 13 days in 2019. From the videos, we counted the number of fruits of different colours (green, yellow or red) before and after each feeding bout in order to calculate the probability of fruit removal in relation to its stage of ripeness. In addition, the time of day, amount of time taken per bout, and animal species and fruit handling behaviour (including seed spitting) were recorded.

Fruit colour

Fruit colour was analysed using an Ocean Optics USB 2000 spectrometer (manufactured by Ocean Insight, Orlando, FL, USA). Using the reflectance probe method described by Johnson and Andersson (2002), we measured spectral reflectance from 300 to 700 nm and established that fruits of both species do not reflect UV light. In the case of *S. puniceus*, we also measured spectral reflectance of unripe, semi ripe and ripe fruits more accurately from 400 to 700 nm using an Ocean Optics DH 2000 light source and ISP 50 8 R GT integrating sphere, which collects all reflected light regardless of the angle of scatter. For *S. multiflorus* subspecies *katherinae*, three fruits of each colour (green, orange and red) were measured. For *S. puniceus*, nine green, 14 orange and nine red fruits were measured. These fruits were collected from different individual plants in the field and were analysed in the laboratory within 12 h of removal from plants.

Dispersal distances

After all fruits on a plant had been dispersed, we located seeds on the ground and measured the dispersal distance (to the nearest cm) using a measuring tape. The dispersal distances were taken from the base of the nearest plant and included every seed found within a 2 m radius around these plants in the case of *S. puniceus* and a 1 m radius in the case of *S. multiflorus*. These radii were chosen as they represent the maximum that was practically feasible for searching given that the leaf layer on the forest floor makes searching for seeds exceptionally laborious. For *S. puniceus*, the number of seeds per plant was counted before dispersal, and for *S. multiflorus*, they were estimated from the mean number of fruits per plant in the population (Butler & Johnson 2020). The number of seeds per plant found within the search radius was then subtracted from the initial number of seeds recorded (or estimated) for that plant, and this number was determined to be the number of seeds that had been potentially dispersed further than the search radius.

Germination experiments

An experiment to determine the effects of pulp removal and fruit ripeness on the timing and proportion of germination of seeds of *S. puniceus* was conducted for 9 weeks within the shade houses in the botanical gardens of the Pietermaritzburg campus of the University of KwaZulu Natal (hereafter referred to as BG; 29°37′27” S, 30°24′15″ E). We used red and green single seeded fruits for the experiments. These either had the exocarp and mesocarp removed by hand or were left unpeeled. As suggested by Robertson *et al.* (2006), both a peeled and an intact treatment were used to test for disinhibition. This allowed results to be compared across colour (green, $n = 35$; red, $n = 117$) and peeling status (peeled, $n = 77$; unpeeled, $n = 75$). Both the unpeeled fruits, that is, intact and unmanipulated fruits, and peeled seeds, which were depulped by hand, were then planted randomly into 20 × 50 mm seed trays and filled with composted soil. Each tray had an equal selection of green and red fruits selected at random, so that there were 10 seeds per tray in total. These trays were then placed in a random order but next to each other in the shade house with additional 40% shade cloth placed over the trays to discourage birds from picking the seeds out of the soil and to simulate the natural shady habitat. In the shade house, a watering system watered the seed trays the same amount at the same time every day. Every week germination, determined by the emergence of the radical from the seed, was recorded for each seed.

Statistical analyses

Analysis of the proportions of fruits removed from plants by monkeys (response variable) in relation to stages of ripeness (independent variable) involved generalized linear mixed effects models (GLMMs) incorporating a binomial distribution (with events/trials design) and logit link

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function, and with plant identity treated as the subject to account for lack of independence among fruits of different ripeness on the same plants. The sequential Šidák method was used for among group multiple comparisons. For the germination experiments, the proportion of seeds germinated (response variable) was analysed using a full factorial binomial GLM (with events/trials design) with time as a covariate and fruit ripeness and peeling as fixed factors. The three way interaction of peeling, fruit ripeness and time, allowed for a different logistic regression slope for each combination of levels in the model. We assessed significance of fixed effects using likelihood ratios in the case of GLMs and F tests based on the Kenward Roger correction in the case of GLMMs.

RESULTS

Animal observations and behaviour

Fruits of both *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* were removed by monkeys and, to a much lesser degree, by birds (Fig. 1; Table 1). The main dispersers were vervet monkeys (*Chlorocebus pygerythrus*), but some dispersal by samango monkeys (*Cercopithecus albogularis*) was evident at KF (Table 1). The monkeys removed fruits (Table 1) with their hands and processed them in their mouth to remove and eat the pulp and then spat out the seeds (Table 1; Fig. 1; Appendix S4). Fruit colour had a highly significant effect on the probability of removal by monkeys for both Scadoxus species (*S. multiflorus* subsp. *Katherinae*: $F = 45.1$, $P < 0.005$; *S. puniceus*: $F = 74.1$, $P < 0.005$). The incidence of consumption by monkeys was much higher for red fruits than for yellow and green fruits (Fig. 2). Fruits at differing stages of ripeness differ in reflectance of visible wavelengths and have negligible UV reflectance (Appendix S1, S2). Birds also selected red fruits and attempted to break open the fruits with their beaks but were usually unsuccessful, giving up before removing much pulp (Appendix S4). Monkeys had a longer handling time (time spent engaging with an infructescence) than birds for both plant species (Table 1). For *S. multiflorus* subsp. *katherinae*, in forested habitats, monkeys and birds visited in the middle of the day, whereas for *S. puniceus* in more open grassland-forest mosaic habitats, monkeys and birds visited mainly in the morning and late afternoon (Appendix S3).

Dispersal distances

Seeds of *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* were mostly dispersed further than 1 m away from their parent plants (Fig. 3). Both species showed some evidence of a decline in dispersal in relation to distance within the search radius (Fig. 3).

Germination experiments

Seeds of *Scadoxus puniceus* had mostly germinated by the end of 9 weeks (Fig. 4). The overall proportion of seeds germinated increased with time from sowing ($\chi^2 = 229.4$, $P < 0.0001$) and pulp removal strongly promoted germination ($\chi^2 = 9.34$, $P = 0.002$; Fig 4). Overall, it took just 2 weeks for 50% of the peeled seeds to germinate and about 5 weeks for 50% of the unpeeled seeds to germinate (Fig. 4). Stage of ripeness ($\chi^2 = 0.040$, $P = 0.842$; Fig 4) and the interaction of ripeness and pulp removal had no significant effect ($\chi^2 = 0.95$, $P = 0.331$), but there were significant interactions between pulp removal and time ($\chi^2 = 5.13$, $P = 0.023$) and pulp removal, ripeness and time ($\chi^2 = 9.36$, $P = 0.002$).

DISCUSSION

Our study reveals that the two *Scadoxus* study species are likely to be specialized for seed dispersal by monkeys, which eat the fleshy parts of the red fruits and spit out the seeds and occasionally the exocarp (Table 1; Fig. 1; Appendix S4). We observed mainly

Table 1. Animal dispersers for *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* and their mean fruit handling times

Plant species	Site	Visitor species	*n*	Mean (± SE) number of fruits taken per visit	Mean (± SE) handling time (s) per infructescence
Scadoxus multiflorus subspecies *katherinae*	VC	Vervet monkey	34	6.9 ± 0.99	93.0 ± 15.00
		Black bellied starling	3	1.0 ± 0.00	3.0 ± 0.00
		Dark capped bulbul	6	0.0	12.0 ± 1.00
		Terrestrial brownbul	6	1.0 ± 0.00	21.0 ± 3.00
		Yellow bellied greenbul	9	1.7 ± 0.17	35.0 ± 3.00
Scadoxus puniceus	KF	Samango monkey	6	4.8 ± 0.61	10.0 ± 1.00
	VC	Vervet monkey	30	5.9 ± 1.26	32.0 ± 3.00
		Dark capped bulbul	7	1.0 ± 0.00	2.0 ± 0.00

KF, Karkloof Forest; VC, Vernon Crookes Nature Reserve.

Fig. 2. Fruit colour preference of monkeys, as reflected in the proportions of available fruits at different ripeness stages that were consumed. (a) *Scadoxus multiflorus* subsp. *katherinae* and (b) *S. puniceus*. Symbols show means ± standard error. Means that share the same letters are not significantly different.

vervet monkeys feeding on *Scadoxus* fruits, in contrast to Kiepiel and Johnson (2019) who found that samango monkeys were the main dispersers of seeds of *Clivia miniata*, another member of the Haemantheae, which has fruits and seeds that are similar to those of *Scadoxus*. This difference in monkey species may reflect that the two *Scadoxus* species studied here occur in relatively open forest grassland mosaic habitats preferred by vervet monkeys, while *Clivia miniata* occurs in homogeneous forest habitats preferred by samango monkeys.

Primate seed spitting as a dispersal mechanism is usually associated with shorter seed dispersal distances of only a few meters. Lambert (1999) found that chimpanzees and redtail monkeys tended to spit seeds less than five meters from where they found the fruit. For the tree *Strychnos mitis*, redtail monkeys would most often move away from the plant before spitting out the seeds, although usually still under the tree crown (Lambert 2001). Kiepiel and Johnson (2019) hypothesized that cheek-pouching may result in occasional long-distance dispersal but were not able to estimate the number of seeds dispersed beyond their search perimeter of 11 m and thus may have underestimated the prevalence of long-distance dispersal. By counting all seeds on plants prior to dispersal in this study, we calculated that for both of our study species, the great majority of seeds were dispersed further than our search perimeter (84% greater than 1 m for *S. multiflorus* subsp *katherinae* and 79% greater than 1 m (and 78% greater than 2 m) for *S. puniceus*) (Fig. 3).

We argue that dispersal of *Scadoxus* seeds by a combination of short-distance spitting and longer distance cheek-pouching followed by further spitting may maximize the chance of dispersal to favourable habitats. Both species are specialized for habitats that are often just a few square meters in area; *S. multiflorus* subsp *katherinae* in swampy, riverine forested habitats and *S. puniceus* in bush clumps or forest patches surrounded by grasslands. Movements of monkeys are dictated by fear of predators, so that monkeys in forest-grassland mosaics actively avoid grassland (an unfavourable habitat for germination of *Scadoxus* seeds) unless they have to move from one forest patch to another. They also spend less time in smaller forest patches than in larger homogenous forest landscapes (Makin *et al.* 2012). If disturbed or if the rest of the troop moves on, the monkeys keep seeds in their cheek pouches when they leave the site, which they then presumably spit out further away (Lambert 2002; Smith *et al.* 2008). If animals spend more time in bush clumps than in the open grassland, this could result in a further, directed, seed dispersal distance as they are unlikely to process fruits in their mouth while moving rapidly across open grassland (Wenny 2001; Makin *et al.* 2012). The seemingly advantageous combination of local and metapopulation dispersal by monkeys in the *Scadoxus* system is a phenomenon that is more typically associated with complementary dispersal arising from different vectors (Spiegel & Nathan 2007; Schupp *et al.* 2010).

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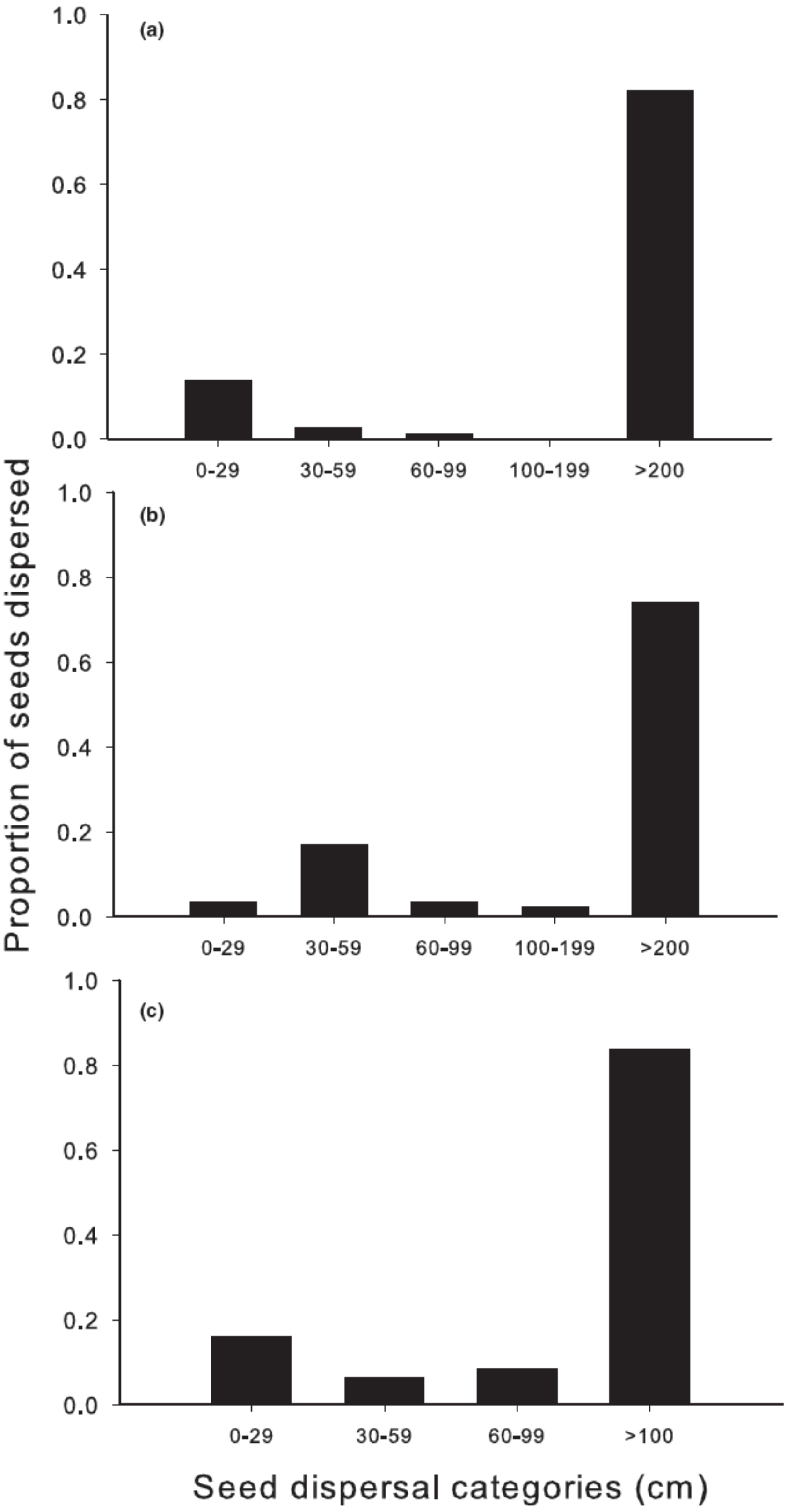

Fig. 3. Seed dispersal distances for *Scadoxus puniceus* at sites (a) KF and (b) VC (see text for more detailed site descriptions), and (c) *S. multiflorus* subspecies *katherinae*.

Our germination experiments for both species revealed that, although monkeys chose red over green fruits, stage of fruit ripeness had no significant effect on germination; however, removal of the fruit pulp sped up the rate of germination (Fig. 4). It should be kept in mind that the green fruits used in this

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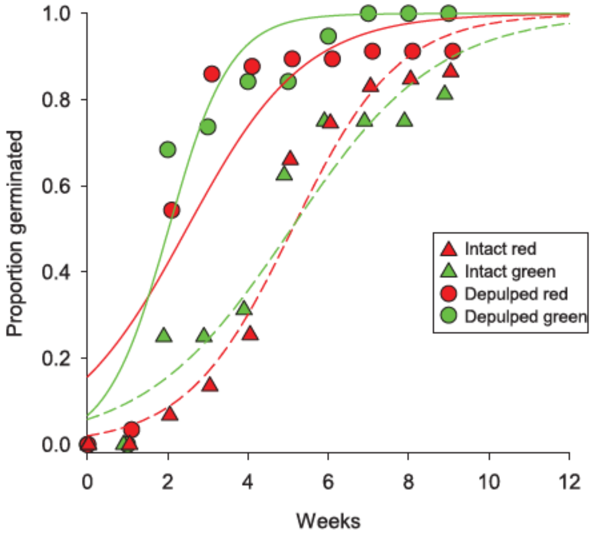

Fig. 4. The cumulative proportion of germinated *Scadoxus puniceus* seeds over time according to stages of fruit ripeness and fruit pulp removal. Red triangles = intact red fruits, red circles = depulped red fruits, green triangles = intact green fruits, green circles = depulped green fruits.

experiment were close to ripening (they were taken from infructescences where some fruits had already turned red) and therefore that these germination results cannot be extrapolated to fruits, which are in early stages of ripening. However, fruit colour changes, normally indicative of ripening, do not affect the germination of the seeds of many species (Cruz-Tejada *et al.* 2018). Manipulation by monkey seed dispersers does increase the rate of germination of seeds of these two *Scadoxus* species through the removal of fruit pulp; however, it does not affect final germination percentage (Fig. 4). Removal of fruit pulp has been found to promote seed germination for a number of other plant species with seed dispersal systems involving monkey spitting (Yumoto *et al.* 1998; Lambert 2001; Kiepiel & Johnson 2019).

CONCLUSIONS

Monkeys disperse the seeds of two *Scadoxus* species (*S. puniceus* and *S. multiflorus* subsp *katherinae*). Both plant species occur in habitats that are well frequented by monkeys. While fruit ripeness did not affect germination probability, depulping had a strong positive effect on the rate of seed germination. It is likely that related species within the Haemantheae, which have fleshy fruits, such as other *Scadoxus* species as well species in the closely related genus *Haemanthus*, also rely on zoochory (Snijman 1984; Meerow & Clayton 2004). However, some of the species in the Haemantheae occur outside the range of monkeys and it therefore seems likely that other animals, such as birds and baboons, are also involved in dispersing seeds of species in this plant group.

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AUTHOR CONTRIBUTIONS

Hannah Claire Butler: Conceptualization (equal); data curation (equal). **Steve Johnson:** Supervision (lead).

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may/can be found online in the supporting information tab for this article.

Appendix S1 Spectral reflectance of fruits of *Scadoxus multiflorus* subspecies *katherinae*. Coloured lines indicate means.

Appendix S2 Spectral reflectance of fruits of *Scadoxus puniceus*. Solid lines indicate means.

Appendix S3 The time of day that A, monkeys visit *Scadoxus multiflorus* subspecies *katherinae* infructescences. B, that birds visit *S. multiflorus* subsp *katherinae* infructescences. C, that monkeys visit *Scadoxus puniceus* infructescences. And D, that birds visit *Scadoxus puniceus* infructescences.

Appendix S4 Video showing dispersal of *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* by monkeys and birds.

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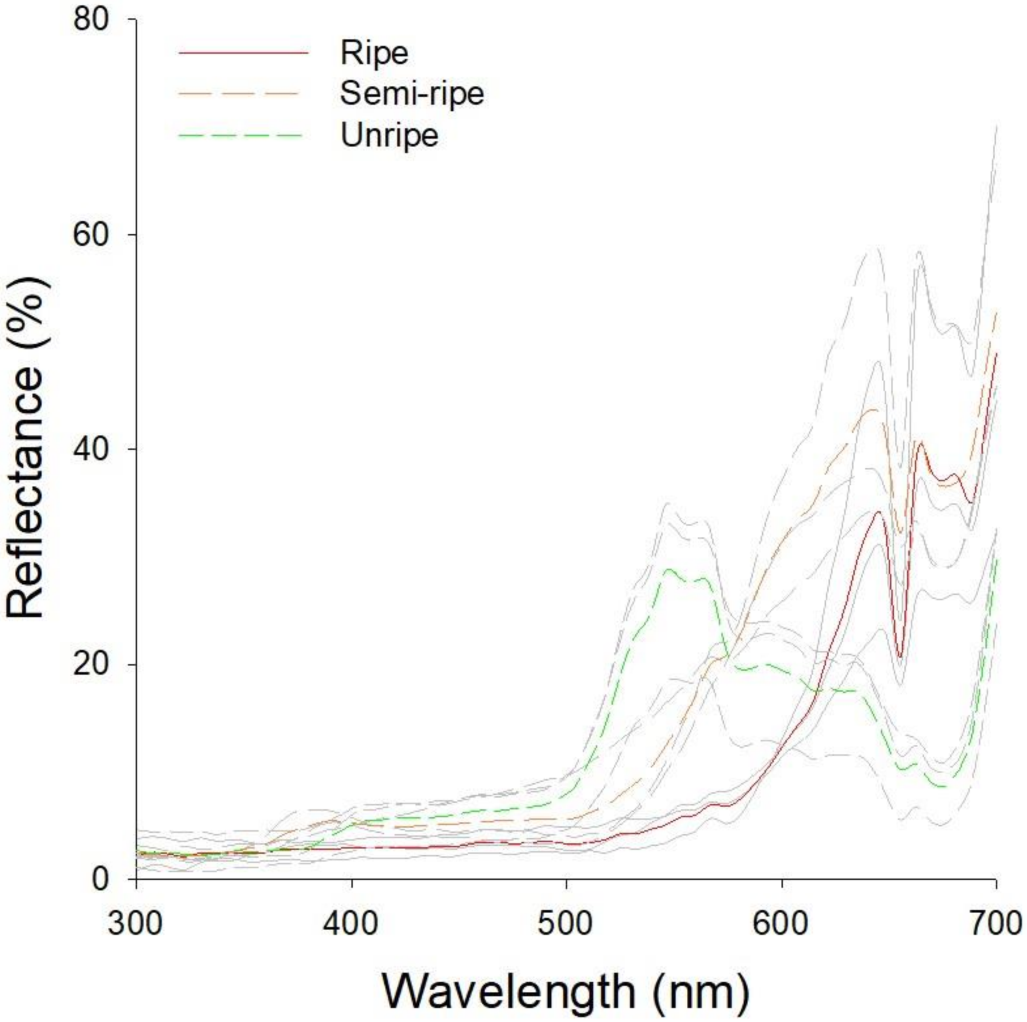
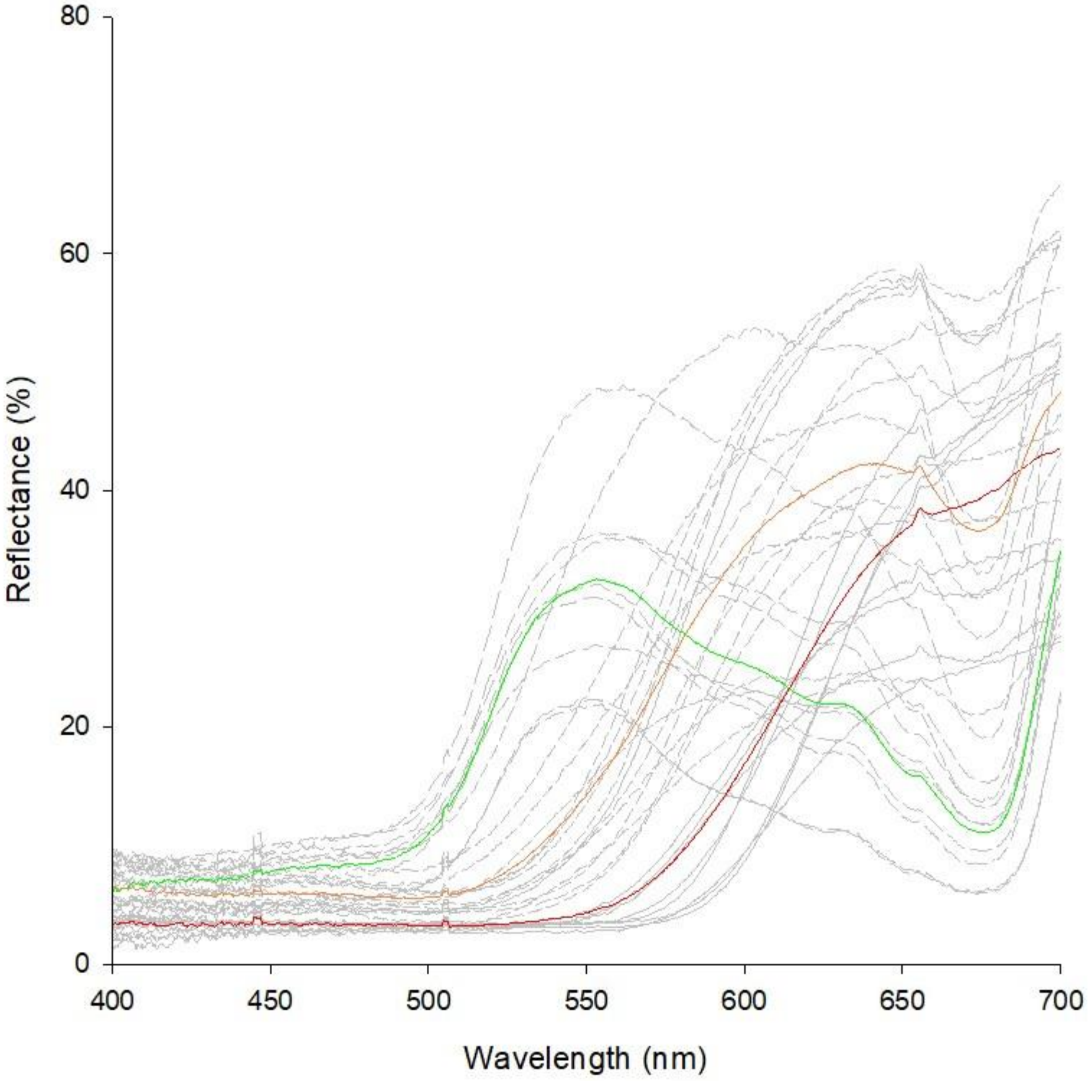
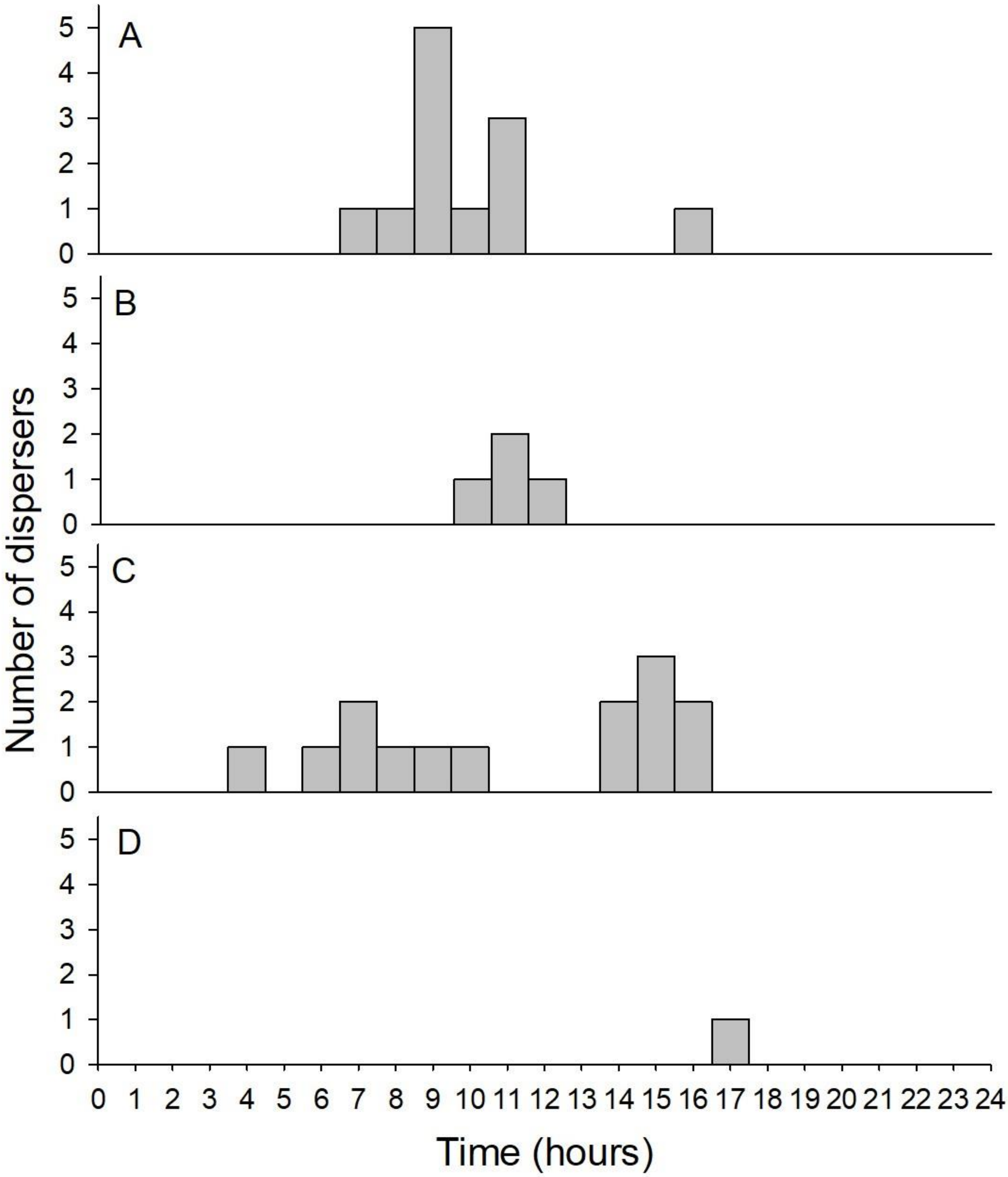
Supplemental figures and tables



Supplemental Figure 1. Spectral reflectance of fruits of *Scadoxus multiflorus* subspecies *katherinae*. Coloured lines indicate means.



Appendix S2. Spectral reflectance of fruits of *Scadoxus puniceus*. Solid lines indicate means.



Appendix S3. The time of day that A) monkeys visit *Scadoxus multiflorus* subspecies *katherinae* infructescences. B) that birds visit *S. multiflorus* subsp *katherinae* infructescences. C) that monkeys visit *Scadoxus puniceus* infructescences. And D) that birds visit *Scadoxus puniceus* infructescences.

Supplemental video 1. Dispersal of *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* by monkeys and birds.

https://drive.google.com/file/d/1f4W6hlXFnA48SiBkFEmnLhSOOf_QvA9o/view?usp=sharing

CHAPTER 6: DIRECTES VERTEBRATE-MEDIATED SEED DISPERSAL OF A FLESHY-FRUITED AMARYLLID IN A HETEROGENEOUS HABITAT

Abstract

Most plants with fleshy fruits have seeds that are ingested by animals, but a less well understood mode of seed dispersal involves fleshy fruits containing seeds that are discarded by frugivorous animals as they are too large or toxic to be ingested. We studied the seed dispersal biology of *Haemanthus deformis*, an amaryllid lily species found in a mosaic of bush clumps in a grassland matrix in South Africa. We asked whether seed dispersal is directed in and among bush clumps and whether germination and survival are greater for seeds dispersed to bush clumps than to those dispersed into grassland. Using camera trapping, we found that fruits are consumed mainly by birds and rodents. The pulp was removed from the seeds which were then discarded without ingestion. Whilst many seed were dispersed close to the parent plant, most (c. 78.5%) were dispersed further than one meter away from the parent plant. Longer distance dispersal resulted mainly from birds flying off with seeds in their bill or from rodents engaging in scatter-hoarding behaviour. Seed germination was most successful within bush clumps as compared to grasslands and shade was identified as a primary requirement for seedling survival. Seeds from which the fruit pulp had been removed germinated faster than those in intact fruits. *Haemanthus deformis* deploys a system of directed seed dispersal, whereby both birds and rodents contribute to dispersal of seeds within patchy bush clumps that are favourable for seedling survival.

Keywords: Bird dispersal, frugivory, germination, post-dispersal factors, rodent dispersal.

Introduction

Seed dispersal is a key process, contributing to the spread and abundance of plant species and, ultimately, the biotic diversity in communities (Cain et al., 2000; Cousens et al., 2008). The seed dispersers of plants are important for the distances of seed dispersal and the microhabitat in which seeds land, both of which influence the chances of successful recruitment (Howe and Miriti, 2004). The effectiveness of a seed disperser depends on the quality of the seeds dispersed as well as that provided for each dispersed seed (Schupp, 1993). Seed dispersal kernels (the probabilities of their dispersal distances) affect abundance, diversity and distribution, which in turn are affected by habitat structure (Levine and Murrel, 2003). Dispersal kernels need to correspond to the spatial structure of the habitat patches required for seedlings to survive and in some cases, this may actually favour short distance dispersal (Mathius et al., 2001).

Plants with seeds dispersed by frugivorous birds and monkeys are usually characterised by fleshy, brightly coloured fruits (Eriksson, 2016; Gautier-Hion et al., 1985). Seeds of these fruits are usually ingested by the frugivores (endozoochory), but there are also cases of stomatochory (seed discarding) and synzoochory (seed caching) where seeds are dispersed without ingestion either during or after the fruit pulp is eaten (Baños-Villalba et al., 2017; Gómez et al., 2018). The latter forms of dispersal are not as well studied as endozoochory, stomatochory and synzoochory have been extensively studied. Key questions about stomatochory and synzoochory remain however and include the identity and behaviour of animal dispersers and the effects of dispersal distances and fruit processing on seed germination. Identification of frugivores is difficult when they are sensitive to human disturbance or active during the night. One solution to this problem is camera trapping which is a highly effective tool for identifying frugivores and for studying their behaviour (Butler and Johnson, 2022; Kiepiel and Johnson, 2019; Mokotjomela et al., 2014; Prasad et al., 2010).

Frugivores manipulate fruits and seeds in many ways, such as dropping, masticating, swallowing and defecating, regurgitating or spitting (Lambert, 2002). Many seeds have adaptations, such as hard seed coats and toxicity, that protect them from destruction by frugivores (Corlett and Lucas, 1990). Seeds that are swallowed are likely to travel further, but in some cases plants may benefit from zoochory that does not involve ingestion (Levey, 1987). Advantages include lack of damage to seeds, as in the case of recalcitrant seeds that lack a hard coat and are thus unable to survive gut passage (Kiepiel and Johnson, 2019). In general, non-ingestion of seeds in fleshy fruit leads to short distance dispersal (Lambert, 1999; Levey, 1987; Yumoto et al., 1998). Long distance dispersal of non-ingested seeds can, however, occur through mechanisms such as cheek pouching in monkeys and birds carrying whole fruit in their mouths or bills (Baños-Villalba et al., 2017). Primates are well known for dispersing seeds via seed spitting, although some ungulates have also been found to engage in this behaviour when they discard seeds while ruminating (Delibes et al., 2019). Fruit-processing by frugivores, such as removal of the endocarp, may also have an impact on germination and seedling recruitment (Lambert, 2002; Rowell and Mitchell, 1991). Removal of pulp from seeds, for example, has been shown to increase germination rates (Lambert, 2001).

The African tribe Haemanthae is the only lineage within the Amaryllidaceae containing species with brightly coloured berry-like (baccate) fruits (Meerow and Clayton, 2004; Rasmussen et al., 2006). Snijman (1984) speculated that lengthening of the peduncle or the movement of seeds along seasonal watercourses are the most likely modes of dispersal in some members of this lineage. However, fleshy fruits are suggestive of specialised interactions with frugivores (Howe and Smallwood, 1982; Snow, 1981). This has been confirmed by some recent studies in the Haemanthae; seeds of *Clivia miniata* were recently found to be dispersed via monkey spitting behaviour (Kiepiel and Johnson, 2019) and *Scadoxus multiflorus* and *S. puniceus* have also been found to share this seed dispersal method (Butler and Johnson, 2022).

Here we address the dispersal biology in *Haemanthus deformis* which has fruits and seeds similar to those in *Clivia* and *Scadoxus*. Because the fruits of *H. deformis* are much softer than those of *Clivia* and *Scadoxus* we predicted that birds would be the primary seed dispersers. The recalcitrant seeds of *H. deformis* lack a hard seed that would enable them to survive gut passage, are very large (c. 10 mm in diameter) and are probably toxic, given the prevalence of seed toxicity in related amaryllids (Nair and van Staden, 2022). We thus predicted that seeds would be discarded without being ingested. Fruit maturity is often indicated by a change in fruit colour, (Schaefer et al., 2007; Schaefer and Schaefer, 2006), and we thus predicted that a preference for a certain colour would correlate with higher germination probabilities for seeds in fruits at that developmental stage.

The aims of this study were to determine: 1) the agents that disperse seeds of *H. deformis* and their behaviour in terms of fruit selection; 2) the distances of seed dispersal; 3) the impact that microhabitats have on germination; and 4) the effect that fruit ripeness and depulping may have on germination.

Methods

1. Study species and sites

Haemanthus deformis Hook.f. inhabits bush clumps in the savanna biome in eastern South Africa (Snijman, 1984). The plants produce two large flat leaves that die and grow back every year and have inflorescences with a very short (25-50 mm) peduncle. The species has a compact inflorescence of white flowers and is pollinated by sunbirds (Butler and Johnson, in press). Fruits are initially green and then become orange when ripe and fruit for approximately one month. Fruits are 15mm in diameter, are soft and pulpy, and have pale seeds which are 28.1 mm ± 0.4 in diameter (n=104 seeds). The mean (± SD) number of fruits per individual plant is 4 ± 5 (n = 28 plants) and seeds per fruit is 1.70 ± 0.09 (range = 1-5, median = 1, n= 18 fruits).

The study was conducted at three field sites; Cumberland nature reserve, Msini private farm in Cato Ridge, and Vernon Crookes nature reserve (map and coordinates of these sites given in Figure S1): Plants at the Cumberland and Vernon Crookes sites occur in scattered bush clumps while those at

Msini occur in more continuous woodlands. Three populations were selected to reflect the heterogeneity of habitats that the species can inhabit. Greenhouse experiments were also conducted.

2. Frugivores

To determine the identity of frugivores which may disperse seeds, we used Bushnell Nature View camera traps (model 1197740, Bushnell Corporation, Kansas, USA). These motion trigger cameras were mounted on small tripods and were set at distances of 600 mm from inflorescences. The cameras were set up to record day and night at maximum sensitivity, with infrared illumination enabled and a high shutter speed, taking both still photographs and videos of up to a minute in length. Each camera was aimed 60cm away from an individual plant and different plants were used every year. Across the three sites, there were a total of 21 cameras over 709 camera traps days (Cumberland = 65; Misini = 66; Vernon Crookes = 5 days; details in Table S1). Pictures and videos were manually checked for evidence of interactions. For each video obtained that had captured a visit, we noted the time of day of visit, the animal species that visited the infructescence, amount of time taken per foraging bout (the time taken from the first contact with the infructescence to the last contact), as well as colour of fruit consumed and number of green and orange fruits available (in the case of greyscale videos taken at night this could only be determined in some cases if there were colour videos of the same fruit taken earlier on the same day). We also recorded whether the animal departed with a whole fruit or if it was eaten *in situ* and, if so, whether or not seeds were discarded. We looked for evidence for scatter hoarding based on the same animal repeatedly visiting a plant from the same direction at short time intervals. This behaviour is known to occur when rodents move fruit to safer localities (White and Midgley, 2017).

3. Seed dispersal distances

After all fruits on a plant had been removed, focal plants at least two meters from other fruiting plants were selected and identified to measure distance. Seed dispersal distances (to the nearest cm) were measured using a measuring tape up to a 1m radius around each plant. Individual seeds could be identified lying on the soil and were left where found. We have not seen any seeds predated (presumably they are toxic) so this was not a problem in our study. The seed distances were measured once all the seeds in the infructescence had been removed. The number of seeds produced by each uniquely labelled plant (n = 29 at Cumberland in 2019 and n =15 in 2021; n = 19 at Msinsi 2019) was counted prior to dispersal. This could be done without opening the fruits as the large seeds can be felt through the fruit. This allowed us to establish the number of seeds dispersed further than a 1m radius from each plant by subtracting the number of seeds found within the search radius from the number of initial number of seeds per plant recorded (Butler and Johnson, 2022).

4. Effects of habitat on germination

To assess the effect of habitat on seedling survival, we placed seeds either in or out of four bushclumps (Figure S2). Seeds were collected as is from the infructescence and placed lying on the soil as this was closest to what happens naturally. In five bushclumps we placed two replicated pairs, each pair consisting of seven seeds (taken from ripe fruits) placed within the bush clumps and another seven placed in grassland five meters from the edge of the bush clump. Only depulped seeds were used. Seeds were placed c. 5 cm apart from each other within steel cages to prevent disturbance by animals. The proportion of seeds that had germinated and survived as seedlings was scored each week until all had germinated or died.

To further isolate the effects of shading on seed germination and seedling survival, trays with seeds removed from ripe fruits were either placed under thick shade cloth (two layers of 40%) or left uncovered within a shadehouse. Each treatment (covered and uncovered) consisted of 36 seeds spread over three trays. All trays (covered and uncovered) were placed in a shade house with a roof of 40% shade cloth on composted soil. The seed trays were watered the same amount at the same time every day. Germination of seeds for both treatment groups was recorded every week for six weeks. Germination was observed as the emergence of a plant leaf from the seed.

5. Effects of ripeness and peeling on germination

To assess the possible fitness consequences of animals selecting and processing fruits differing in ripeness, we determined the effects that fruit ripeness (orange versus green stages) and pulp removal have on seed germination. Fourteen trays, each consisting of seeds from four unpeeled fruits (two ripe and two unripe) and four peeled fruits (two ripe and two unripe), were planted into a seed tray and filled with composted soil in a greenhouse where they were not moved, and the proportion of seeds that had germinated was scored each week until all had germinated. This was done for 14 trays (total sample = 112 fruits). Fruits from multiple different bush clumps were used.

5. Statistical analyses

The proportion of orange versus green fruits (different ripeness stages) consumed by different animal groups was analysed with generalized estimating equations (GEEs) incorporating a binomial distribution and logit link function. To control for lack of independence among fruits on a plant, plant was treated as the subject and the correlation matrix was exchangeable. Fixed factors were fruit colour, animal type and the interaction of colour and animal type. Significance was based on Score statistics. For the greenhouse and field germination experiments, the proportion of seeds germinated in relation to time was analysed using a full factorial binomial generalized linear model (GLM) with events/trials design. A two-way interaction of proximity to bush clumps (in vs out) and time was used. We assessed significance of fixed effects using likelihood ratios in the case of GLMs. A full factorial gamma generalized linear mixed model (GLMM) with log link function and was used to assess whether the number of weeks taken for seeds to germinate could be predicted by the categorical factors pulp removal (removed or not removed) and fruit ripeness (orange or green). A GLMM with logit link function was used to assess whether the proportion that germinated over time could be predicted by the categorical factors pulp removal (removed or not removed) and fruit ripeness (orange or green). Plant identity and tray number were used as random effects for both tests. A chi-square test was performed to compare seed germination in the shaded versus unshaded treatments. Statistics were implemented using SPSS 24 (IBM corp.).

Results

1. Frugivores

At the Cumberland site, nine bird species and two rodent species were recorded, but only bird species (four) were identified as seed dispersers (Table 1; Figure 1; Video S1). At the Cato Ridge site, a five bird species, three rodent species and one primate species were recorded and one bird species and two mammal species (*Micaelmys namaquensis*; *Graphiurus ocularis*) were identified as seed dispersers (Table 1; Figure 1; Video S1). Vervet monkeys were present at all sites, but the only instance of them consuming fruits was recorded at the Vernon Crookes site (Table 1). Single instances of bushbuck eating an entire infructescence with green fruits were recorded at Cumberland and Cato Ridge. Frugivores consistently chose orange (ripe) over green (unripe) fruits. The proportion of fruits consumed was strongly predicted by fruit colour ($\chi^2 = 27.60$; $p < 0.0001$), but not by animal type ($\chi^2 = 2.93$; $p = 0.231$). The interaction of animal type and colour was not significant and was dropped from the final model. On a per visit basis, the mean (± SE) percentage of available fruits consumed by birds was 37 ± 5.7 % for orange fruits and 0% for green fruits. Monkeys consumed 92 ± 5.2% of orange fruits and 0% of green fruits, while rodents consumed 24± 5.2 % of orange fruits and 0% of green fruits.

Across all sites where cameras were set up, the number of fruits where the pulp was eaten and where fruits were carried beyond the view of the camera were similar for birds and rodents (Table 1). Birds took longer than rodents to process fruits (Table 1). Birds were also seen to 'fling' seeds from their fruits, where, whilst eating the fruits, the birds would shake their heads vigorously, resulting in the large, hard seeds being dispersed close to, but not right next to, the parent plant. Rodents either processed fruit next to the plant by eating fruit surrounding the seeds whilst holding it in their paws, or they carried individual fruits beyond the view of the camera (Table 1). Evidence for scatter hoarding included repeat visits, where a presumed single rodent individual returned to the infructescence at regular time intervals from the same angle, and seeds that were found in groups under rocks where rodents may shelter from potential predators.

Table 1. Camera footage of animals recorded, and animals assumed to be seed dispersers based on those that interacted with the fruits. CR = Cato Ridge, CU = Cumberland, VC = Vernon Crookes.

Site	Visitor type	Visitor species	Individuals	Individuals that removed fruit	Fruits eaten *in situ*	Fruits carried away	Mean (±SE) fruit removed per visit	Mean (±SE) bout length (s)
CR	Bird	*Cossypha caffra* (Cape robin-chat)	1	0	--	--		--
		Pycnonotus tricolor (Dark-capped bulbul)	9	8	3	2	0.63 ± 0.15	57 ± 20
		Camaroptera brachyura (Green-backed camaroptera)	3	0	--	--		--

		Melaniparus niger (Southern black tit)	1	0	--	--		--
		Turtur tympanistria (Tambourine dove)	1	0	--	--		--
	Rodent	*Grammomys dolichurus* (Woodland thicket rat)	2	0	--	--		--
		Graphiurus ocularis (Spectacled dormouse)	3	2	1	1	1 ± 0	23 ± 15
		Aethomys namaquensis (Namaqua rock rat)	43	27	12	8	0.74 ± 0.21	27 ± 5
CU	Bird	*Cossypha caffra* (Cape robin-chat)	1	1	--	1	1	3 ± 0
		Trachyphonus vaillantii (Crested barbet)	4	3	2	--	0.67 ± 0.07	44 ± 10
		Pycnonotus tricolor (Dark-capped bulbul)	17	14	6	3	0.64 ± 0.04	34 ± 10
		Camaroptera brachyura (Green-backed camaroptera)	1	0	--	--		--
		Prodiscus zambesia (Green-backed honeyguide)	7	6	5	1	1 ± 0.05	48 ± 20
		Cisticola fulvicapilla (Neddicky)	13	0	--	--		--
		Laniarius ferrugineus (Southern boubou)	2	0	--	--		--
		Andropadus importunes	3	3	2	1	1 ± 0	16 ± 4

		(Sombre greenbul)						
		Cercotrichas leucophrys (White-browed scrub robin)	10	0	--	--		--
	Rodent	*Grammomys dolichurus* (Woodland thicket rat)	4	0	--	--		--
		Aethomys namaquensis (Namaqua rock rat)	31	0	--	--		--
VC	Monkey	*Chlorocebus pygerythrus* (Vervet monkey)	1	1	1	--	1	19 ± 0

Figure 1. Animal seed dispersers of *Haemanthus deformis*. (A) sombre greenbull (*Andropadus importunes*); (B) cape bulbul (*Pycnonotus capensis*); (C) crested barbet (*Trachyphonus vaillantii*); (D) vervet monkey (*Chlorocebus pygerythrus*) at the Vernon Crookes site; (E), (F) and (G) Namaqua rock mouse (*Micaelamys namaquensis*) at the Cato Ridge site. Red arrows point to seeds. All scale bars represent 50 mm.

2. Seed dispersal distances

Most seeds (c. 78.5%) were dispersed further than one meter (Figure 2). We found 1282 seeds found within the 1m search radius around 69 plants and, on average, 18.58 seeds were found per plant. For seeds that were dispersed within one meter of the parent plant, there was evidence for a leptokurtic pattern with the most frequent category being < 10 cm from the parent plant (Figure 2).

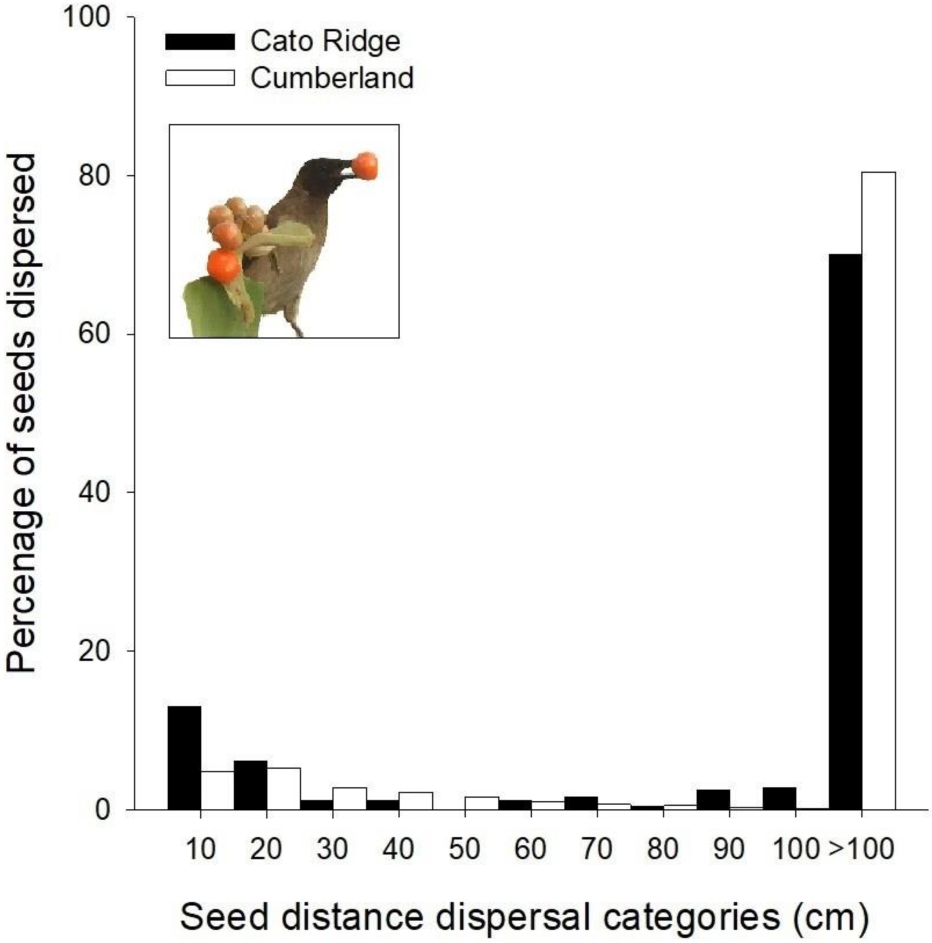


Figure 2. Percent seed dispersal distances displayed in seed dispersal distance categories for *Haemanthus deformis* at two sites, Cato Ridge and Cumberland. Seed distances were measured after dispersal from the found seed to the closest plant.

3. Effects of habitat on germination

Seeds within bush clumps were more likely to survive as seedlings as compared to seeds in the open grassland (F = 14.425; $p < 0.0001$) (Figure 3). Seedlings from one third (12 out of 36) of the seeds placed under thick shade survived after 16 days, while very few (3 out of 36) of the seeds that were not placed under extra shade cloth had survived up to 16 days ($\chi^2 = 6.821$; $p = 0.018$).

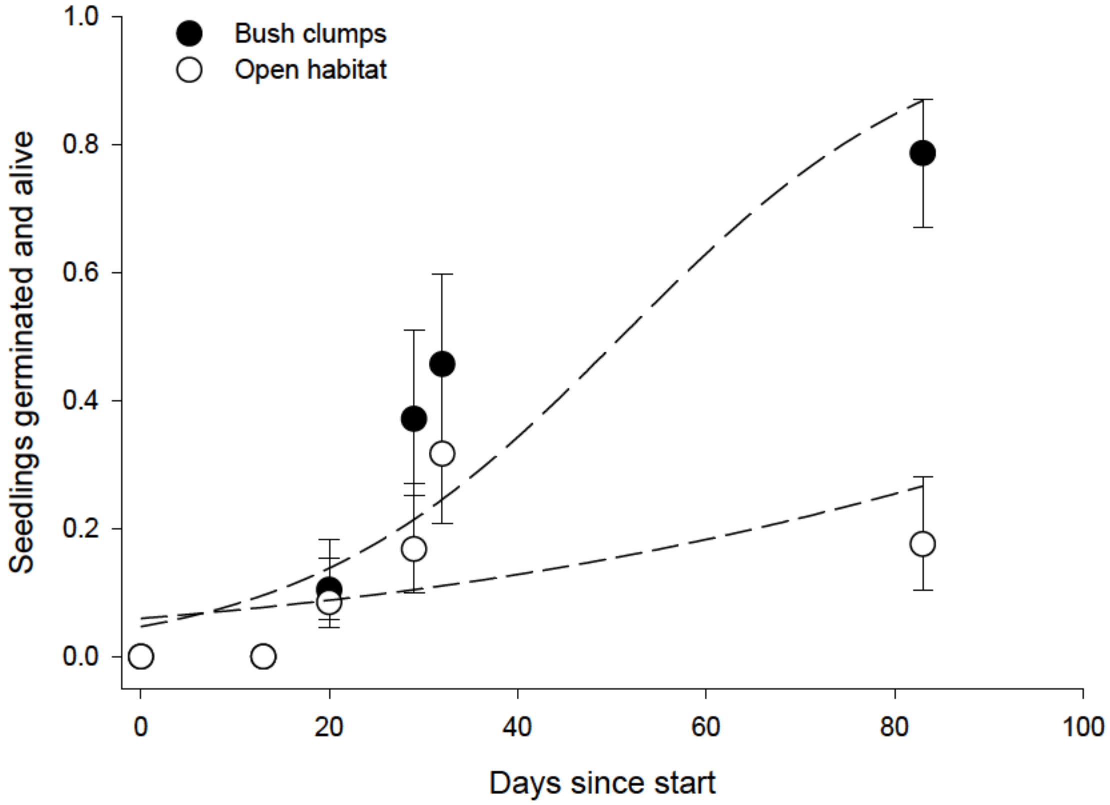


Figure 3. Changes in cumulative proportions of *Haemanthus deformis* seeds germinated and surviving as seedlings in either open habitat or bush clumps.

4. Effects of ripeness and peeling on germination

The proportion of seeds from unripe (green) fruits that germinated after four weeks was lower than for those from ripe (orange) fruits (F = 19.00; $p < 0.0001$), and pulp removal increased germination (F = 6.296; $p = 0.013$; Fig 4a). The interaction of ripeness and pulp removal was non-significant (F =

2.76; p = 0.098). Seeds from green fruits took longer to germinate than those from orange fruits (F = 25.23; p < 0.0001) and seeds from unpeeled fruits took longer to germinate than seeds from peeled fruits (F = 55.09; p < 0.0001; Fig 4b). The interaction of ripeness and pulp removal on the time take for seeds to germinate was significant (F = 11.68; p = 0.001).

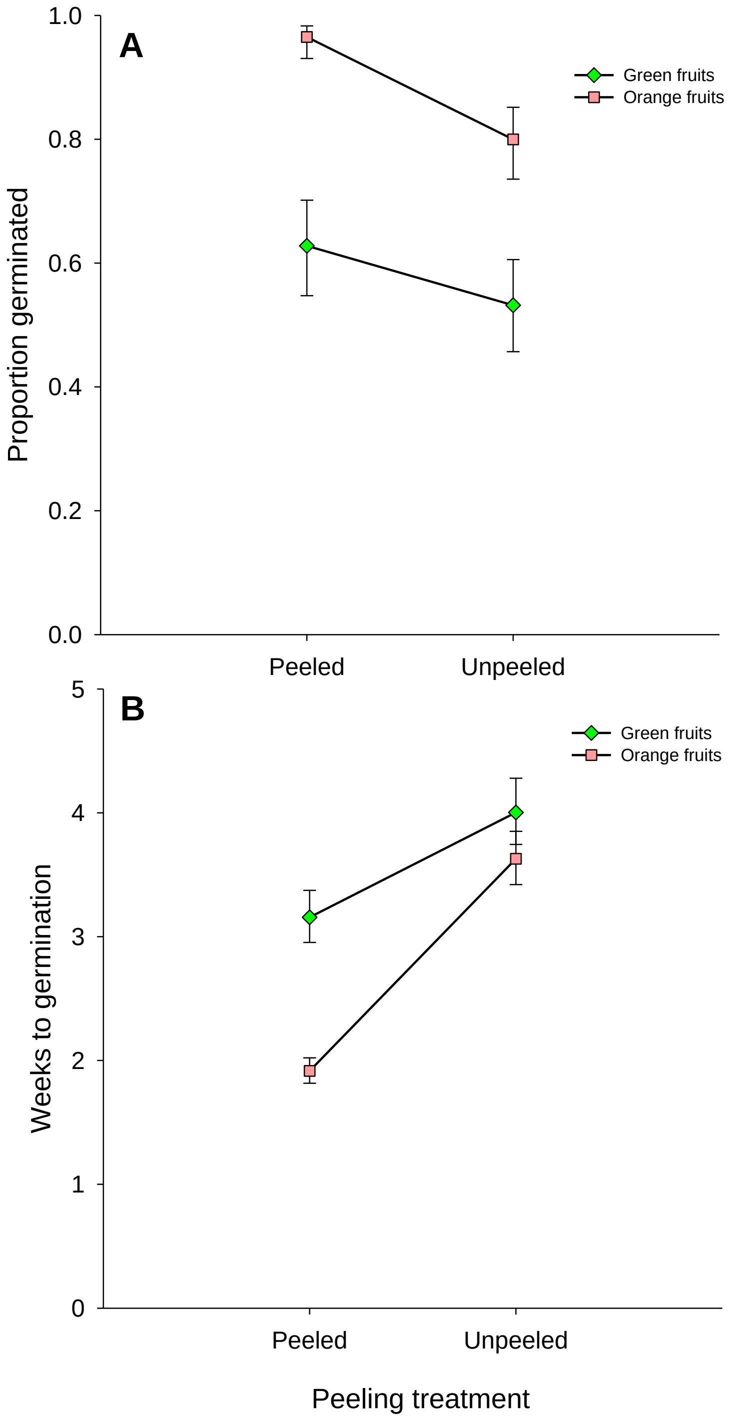


Figure 4. The (A), proportion of germinated seeds and (B), weeks to germination for seeds from unpeeled versus peeled fruits as well as green versus orange fruits (represented as green triangles and orange squares respectively).

Discussion

We found that the primary seed dispersers of *Haemanthus deformis* are birds and rodents (Figure 1; Table 1; Video S1), though there was considerable variation in these assemblages among sites which may relate to differences in vegetation structure. We found that seeds from unpeeled, orange as compared to green fruits, germinated the fastest and that seeds in bush clumps as compared to seeds that were placed in the surrounding savanna survived better. We also found open sun as compared to shade to be an inhibitor of germination.

Both rodents and birds consistently chose orange over green fruits and removed the pulp from the seeds. This behaviour contributed positively to recruitment (Figure 4). Similar results were found for species within the related genera, *Scadoxus* and *Clivia*, all of which are in the tribe Haemantheae, the only Amaryllid tribe to have fleshy berry-like fruits (Butler and Johnson, 2022; Kiepiel and Johnson, 2019). Removing the fruit pulp from the seeds may remove germination inhibitors in the fruit, allowing the seeds to germinate faster and can help to remove fungal and microbial infections in the fruit (Cipollini and Levey, 1997; Evenari, 1949; Lambert, 2002; Levey, 1987). Germination inhibitors may also be more present in greener, younger fruits, so a preference by a frugivore for more brightly coloured fruits that are riper may also allow seeds to germinate faster.

Haemanthus deformis is found within the bush clumps where the species is found as often more than ten plants within a one meter radius. These bush clumps are surrounded by savanna (circa 30m apart) (Figure S2) and many are formed around termitaria which impact various soil and water attributes and therefore vegetation dynamics (Konaté et al., 2006). This leads to a distinct floral composition within bush clumps as compared to the surrounding savanna (Bloesch, 2008). In situ germination experiments showed that de-pulped seeds placed within a bush clump survived better than those placed in the surrounding savanna, suggesting the importance of habitat in the germination and recruitment of the species.

Many birds were seen to ‘fling’ the seeds when eating them (Video S1). Such behaviour caused the birds to successfully remove the seeds from the pulp and then to eat the pulp. Very short dispersal (< 10 cm) is probably disadvantageous due to competition with parent plants, but because some bush clumps are just a few square meters in area, seeds dispersed distances from 10 cm to several meters would have a better survival probability than those dispersed longer distances into the surrounding grassland (Figure 3). Short distance dispersal of further than a few meters between clumps may contribute to the colonization of new areas. Birds that flew off with a fruit in their bills were likely to have processed the fruit in another bush clump. Seeds are unlikely to be dispersed into the surrounding grassland matrix as birds would not process fruits while in flight and rodents would tend to remain within a particular bush clump. In general, frugivory by specialised frugivores such as birds results in longer distances of seed dispersal (Vander Wall and Beck, 2012).

Most seeds are dispersed further than one meter (Figure 2). It is therefore highly likely that colonization of new bushclumps is possible. Directed short-distance dispersal of seeds by birds and rodents maintains colonies in bush clumps, while directed long distance of seeds by birds, and in some cases monkeys, allows for occasional colonization of new bush clumps (Cain et al., 2000; Howe and Smallwood, 1982; Wenny, 2001). It seems that no secondary disperser is involved as no other disperser apart from the birds and rodents were seen interacting with the seeds, and then only interacted when fruit pulp was available.

The fleshy and brightly coloured fruits of species in the amaryllid tribe Haemantheae, including *H. deformis*, confirm to the bird-monkey dispersal syndrome (Gautier-Hion et al., 1985). However, our data suggest that rodents are important dispersers of *H. deformis* seeds at some sites. Videos revealed that rodents ate the fleshy pulp in front of the cameras or would repeatedly carry the fruits beyond view, which could result in a scatter hoarding distribution pattern (Vander Wall and Beck, 2012). Rodents are rarely known to consume fleshy fruit (Lessa et al., 2019; White and Midgley, 2017; Yang et al., 2018). On occasion, we did see heaps of seeds near rodent droppings which is indicative of caching and subsequent consumption of fruits.

Seeds of the Haemantheae are large and are assumed to be toxic on the basis that other plant parts are highly toxic and the seeds are never eaten and are extremely bitter-tasting to humans (Kiepiel and Johnson, 2019; Nair and van Staden, 2022). Studies of related amaryllids have shown that seeds are defended by alkaloids (Moodley et al., 2022). Seed toxicity is the most likely reason why rodents appeared to discard the seeds without consuming them. The seeds are also recalcitrant (lacking dormancy) and typically germinate within 20 days (Figure 4B) and the climate at the sites is subtropical making it unlikely that rodents use the seeds for seasonal caching (Vander Wall and Beck, 2012). It is more likely that fruits containing seeds are removed to places, such as rock crevices, where the fruit pulp can be safely consumed by rodents with lower risk of predation.

The genus *Haemanthus* occurs in different habitats across South Africa (Snijman, 1984). Although all produce fleshy fruits with few large seeds, they vary in colour of fruits, seeds and even fruit scent (*H. humilis* subspecies *hirsutis* has a sickly sweet smell, Butler, pers. obs.), all of which may impact the type of disperser. It is likely that many are dispersed by birds, as the bright colours (pink or orange) of these species don’t generally occur in areas frequented by monkeys (Gautier-Hion et al., 1985; Snijman, 1984).

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Supplemental information

Supplemental video 1. Animal seed dispersers of *Haemanthus deformis*.

https://drive.google.com/file/d/1DWpvz8X9EsBKl5xwlLYT902_Zybjg9UF/view?usp=share_link

Supplemental Table 1. The number of camera traps and days that they were set up for each year and site.

Site	Year	Number of cameras	Number of days
Cumberland nature reserve	2019	6	20
	2020	9	20
	2021	6	25
Msini private farm	2018	4	17
	2019	4	39
	2020	9	20
Vernon Crookes nature reserve	2018	1	5
Total		39	709

Supplemental Figure S1. (A) Aerial map showing the three sites where experiments were carried out, with the city of Pietermaritzburg as reference. Scale bar represents 40km. (B) Close up aerial map of the Cumberland Nature Reserve. Individual bush clumps used in the study are indicated with red dots. Scale bar represents 800m. (C) Habitat shot of Cumberland Nature Reserve site showing a mosaic landscape of grassland and bush clumps.

CHAPTER 7: DISCUSSION

Summary of results

The aims of this study were to determine the pollination, breeding, and seed dispersal systems of species of *Scadoxus* and *Haemanthus* (Amaryllidaceae).

In chapter 2, I examined the pollination system of *Scadoxus multiflorus* and determined that two subspecies, *multiflorus* and *katherinae*, are pollinated via butterfly wings. The morphology of the flowers is adapted to this system, with exerted stigmas and anthers and pronounced herkogamy. This ensures that, when a butterfly approaches and drinks nectar, their fluttering wings contact the reproductive parts. I also show that many other species of South African Amaryllidaceae are likely to be pollinated via butterfly wings. This is based on scientific and anecdotal evidence and revealed that all the flowers that fitted this new syndrome had similar floral morphology and could be separated into 'open-brush' flowers (for example, *S. multiflorus*) or 'bowl-brush' flowers (for example, *Clivia miniata*). These species are *S. multiflorus*, *Nerine sarniensis*, *Brunsvigia marginata*, *Clivia miniata*, *Cyrtanthus elatus*, *Cyrtanthus flamossus*, *Cyrtanthus guthrieae*, *Cyrtanthus montanus*, and *Cyrtanthus taitii*.

In chapter 3, I determined the pollination and breeding systems of *Scadoxus puniceus* and *S. membranaceus* and found them to be adapted to sunbird pollination. Both species have 'paintbrush' inflorescences, which are made up of many individual flowers that are closely packed together. Each flower is red, contains nectar and is cosexual with low levels of herkogamy. *S. puniceus* is visited frequently by sunbirds and honeybees, and caging experiments revealed that sunbirds were the primary pollinators as fecundity declined markedly when birds were excluded from plants. *S. membranaceus*, however, was hardly visited by animals and only two visits by sunbirds were recorded despite extensive camera trapping and direct observations. This species was found to be self-compatible and even set some fruit autonomously. This contrasts with *S. puniceus* which is self-incompatible.

In chapter 4, I investigated four species of *Haemanthus*, *H. albiflos*, *H. coccineus*, *H. deformis*, and *H. humilis* subspecies *hirsutis*, and determined that sunbirds visited all species. Caging experiments revealed that *H. albiflos*, *H. coccineus* and *H. deformis* were dependant on sunbird pollination, while this was not the case for *H. humilis* subsp. *hirsutis* which was effectively co-pollinated by insects. All these *Haemanthus* species have paintbrush-shaped inflorescences, with individual flowers fitting the bird pollination syndrome. *H. coccineus* has red flowers, and *H. albiflos* and *H. deformis* have white flowers. This may reflect that habitat, as white flowers may be more conspicuous to birds than are red flowers in dark forest habitats. *H. humilis* subsp. *hirsutis* has a pale pink flower colour and attract, as well as sunbirds, *Philoliche* flies and *Amegilla* bees, seen occasionally through direct observations.

In chapter 5, I studied fruit selection, dispersal distances and effects on seed germination in the monkey-spitting seed dispersal systems of *S. multiflorus* subsp. *katherinae* and *S. puniceus*. Both species produce bright red fruits when mature and the monkeys preferentially chose the red fruits over the immature green fruits. Germination experiments showed that seeds from red fruits germinated faster than seeds from green fruits. The monkeys, when interacting with the fruits, peeled the seeds of the succulent pulp and ate the pulp, spitting out and discarding the seeds either immediately next to the plant, or more often, a further distance away after keeping the seeds in their cheek pouches. The germination experiments showed that seeds that had been peeled germinated faster than seeds that had not been depulped.

In chapter 6, I examined the seed dispersal system of *Haemanthus deformis*, which involved both birds and rodents and resulted in directed dispersal to favourable, shady environments where germination was higher. This species occurs in bushy savanna, different from the *Scadoxus* species studied, which occur in forested habitats. Monkeys are common in the habitat in which *H. deformis* occurs. However, at one site, rodents were the most prevalent disperser, while at another, birds were more prevalent. Both dispersers ate the fruits on camera, dispersing the seeds in the immediate vicinity of the parent plant, or, most often, carried the fruits further away to eat them further than the view of the cameras. Greenhouse experiments showed that depulped seeds from mature orange fruits germinated the fastest. In situ experiments showed that seeds that were placed inside of a bush clump produced more surviving seedlings than those outside of bush clumps. Further greenhouse germination experiments showed that shade was the important factor for seed survival.

Pollination and potential for pollinator shifts in *Scadoxus*

Floral diversity is often correlated with pollinator diversity, and plant speciation can result from a shift in pollinator (Fenster et al., 2004; van der Niet and Johnson, 2012). This is often accompanied by floral trait modifications. Floral morphology is correlated with a difference in pollinators in the amaryllid genus *Scadoxus*. Evidence from this study show that *S. multiflorus* is pollinated via butterfly wings, and *S. puniceus* is pollinated by sunbirds (Chapter 2 and 3, Figure 1). Although *S. membranaceus* is rarely visited and can set fruit and seeds autonomously, it does appear to be adapted to pollination by sunbirds given the similarity in floral morphology with *S. puniceus* (Chapter 3).

The *Scadoxus* species discussed here have evolved inflorescence and floral traits that fit their pollinators. For *S. multiflorus*, the inflorescence is a lax umbel, and the flowers are an open ‘brush’ style with pronounced herkogamy. This allows and facilitates pollination via butterfly wings. The inflorescence and flowers do not provide a landing platform for birds, or for their butterfly pollinators, forcing them to flutter when drinking the nectar provided. During wing fluttering, pollen transfer happens from plant to wing. The short distance between stigmas of different individual flowers via the overlap of the flowers in the inflorescences allow for pollination between individual flowers, increasing outcrossing.

The species investigated here that have paintbrush inflorescences are all pollinated by sunbirds (Chapter 3 and 4). A paintbrush inflorescence is an umbel made of tightly packed cosexual flower and facilitates bird pollination by having nectar as a reward and long tubed flowers. The length and narrow tube of the flowers does not allow for other visitors, such as honeybees, to successfully get to the nectar. The inflorescence of *S. puniceus* forms a large head and has a thick peduncle. This allows sunbirds to use the inflorescence itself as a perch when feeding. Due to the nature of inflorescence which has many flowers that are packed together, one can view the inflorescence as a single flower from afar, where the flowers collectively attract a pollinator which visits the flowers individually.

All species investigated, for both *Scadoxus* and *Haemanthus*, have nectar as a reward (Chapter 2, 3 and 4). The amount of nectar produced per flower is relatively small compared to other bird pollinated species. However, given that the inflorescences have up to 100 or more flowers, this simply encourages birds to visit several flowers. Although other insect pollinated plants provide nectar as a reward, the tube length of *Scadoxus* and *Haemanthus* is long and narrow which does not allow visiting honeybees to reach the nectar, but instead places pollen either on the wings of a butterfly or on the feathers surrounding the beak of a sunbird.

The molecular phylogeny of *Scadoxus* of Bødker (2020) showed *S. membranaceus* and *S. puniceus* to be polyphyletic and the relationship between them unresolved, despite obvious morphological characteristics, and ecological differences as discussed in Chapter 3. *Scadoxus puniceus* by itself is not monophyletic and Moe (2020) suggested that *S. puniceus* should be separated into three species. The phylogeny of the genus *Scadoxus* shows strong geographic patterns, and *S. multiflorus* in particular has a large range. On the basis of the molecular data, *Scadoxus multiflorus* subsp. *katherinae* can be separated from *S. multiflorus* subsp. *multiflorus* and appears to be sister to *S. membranaceus*.

The pattern of trait differences of open brush (for example, *S. multiflorus*) and closed brush (for example, *S. puniceus*) flowers identified between the two studied species also characterise all other *Scadoxus* species' floral morphologies. Both Nordal and Duncan's (1984) and Bodker's (2020) phylogeny demonstrate dominant bracts to be derived, and suggest that the ancestral form of *Scadoxus* has less showy bracts (like the widespread *S. multiflorus*). Dominant bracts could be seen as a characteristic of bird pollination, as they keep the flowers tightly packed together in a paintbrush inflorescence which is used by birds as a perch. One can therefore hypothesise that butterfly pollination is ancestral. I therefore hypothesise that all nine species of *Scadoxus* are either pollinated by sunbirds or by butterflies and tentatively suggest three pollinator shifts from butterfly to bird pollination (potential bird pollinated species: *S. cyrtanthiflorus*; *S. puniceus* and *S. membranaceus*; and *S. nutans*) (Figure 17 in Bødker (2020)) (*S. cyrtanthiflorus* has quite different flowers, which are distinct from each other and hang down, much like the bird-pollinated *Clivia* species).

Pollination via butterfly wings appears to be common in the South African Amaryllidaceae and could be an important ancestral trait in *Scadoxus* (Chapter 2). As shown in Chapter 2, only larger butterflies effectively contact the reproductive parts of the flowers and initiate pollination. Mertens et al. (2020) found butterfly pollination for *S. cinnabarinus* in Mount Cameroon, but no other studies have been done on *Scadoxus* pollination. Butterfly wing pollination is relatively rare, but founds in several plant families, including lilies (for example: *Lilium philadelphicum* (Harder and Schowalter, 2022), *Lilium leucanthum* (Liu et al., 2022), and *Lilium margaton* (Corbera et al., 2018)).

Intriguingly, in the related genus *Clivia*, Kiepiel and Johnson (2014b) discovered a shift from bird to butterfly wing pollination – exactly the opposite of what is proposed here. Trait modifications associated with a shift in pollinator must follow the "line of least resistance" as described by Stebbins (1970). Both bird and butterfly pollinated plants have long corolla tubes, which may facilitate a shift between the two. For *Scadoxus* as well as for *Clivia*, the shift appears to be associated with a change in herkogamy. Shifts between butterfly and birds are however scarce but this may be under reported (van der Niet and Johnson, 2012).

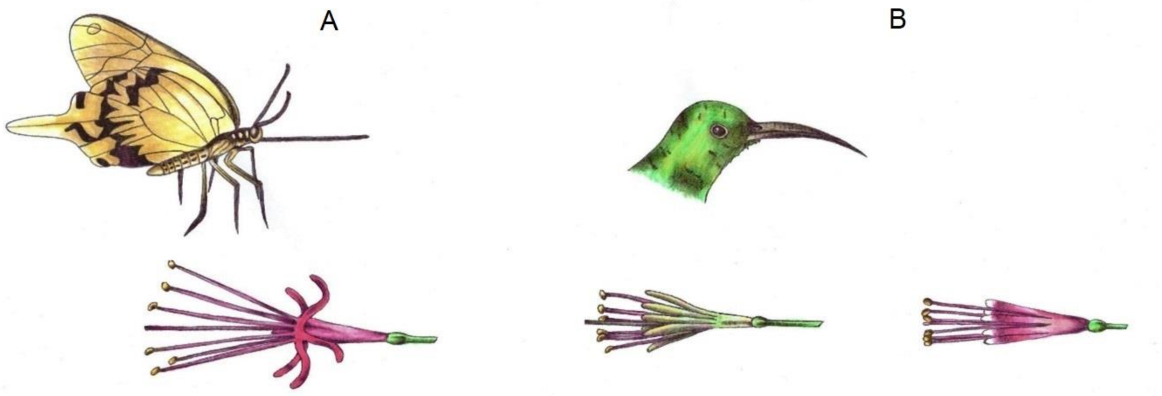

Figure 1. Flowers of two species of *Scadoxus* and their pollinators. A) *Scadoxus multiflorus* flower and mocker swallowtail butterfly; (B) *Scadoxus puniceus* flowers (two morphs) and double-collared sunbird. Drawings: HC Butler.

Implications of breeding systems

Breeding systems are critical in our understanding into the life history of a plant and impact evolution (Charlesworth, 2006). They predict the extent to which selfing can occur and therefore the level of outcrossing required for successful reproductive output. This points to the huge impact on the importance that pollinators play, by increasing selfing and outcrossing. Often, plants evolve various mechanisms to decrease selfing and increase outcrossing as selfing can have detrimental effects on plants, such as inbreeding depression (Charlesworrth and Charlesworth, 1987). These mechanisms are in the shape of floral modifications, which dictate the type and behaviour of visiting pollinators (Ornduff, 1969). Selfing however does have its advantages, such as maintaining genes and removing the impact that a paucity of pollinators in the environment may have on reproduction, and there are many plants that have evolved floral modifications solely for selfing (Lloyd and Schoen, 1992; Schoen et al., 1996). However, some plants capable of selfing may still have a morphology suited to and associated with high levels of outbreeding (Cozien, 2021; Sun, 1997).

Phylogeny and pollination system often predicts breeding system, although that does not seem to be the case here as the self-compatible *S. membranaceus* appears to be adapted for the same pollinators as the self-incompatible *S. puniceus*. This may be to increase the number of visits by pollinators to increase outcrossing although *S. membranaceus* appears to be rarely visited. It is likely that the self-compatibility of *S. membranaceus* has evolved to cope with a paucity of sunbird pollinators. It seems likely that ancestors of Haemanthinae were self-incompatible and that self-compatibility has evolved more recently for some species.

Breeding system analysis of *Scadoxus multiflorus* subspecies *katherinae* have revealed a system of late-acting self-incompatibility (LSI), where self-pollen is rejected only in the ovules (Chapter 2). For *S. puniceus*, a system of self-incompatibility was also revealed (Chapter 3). Intriguingly, *S. membranaceus*, which is closely related to *S. puniceus*, is self-compatible (Chapter 3). The *Haemanthus* species studied here (*H. coccineus*, *H. deformis*, and *H. humilis* subsp. *hirsutis*) are likely to be self-incompatible as flowers from which pollinators were excluded produced few fruits, despite the high likelihood of anther-stigma contact (Chapter 4).

A shift from xenogamy to autogamy has occurred many times and across a range of taxa within flowering plants (Allen and Hiscock, 2008). Within the Amaryllidaceae, Johnson et al. (2019) found LSI in another South African amaryllid genus, *Cyrtanthus contractus*, but also discovered self-compatibility for *C. mackenii*. Other systems of LSI in South African amaryllids include *Cyrtanthus breviflorus* (Vaughton et al., 2010) and *Clivia* (Kiepiel and Johnson, 2014a). Variation in self-compatibility is also present in another, well studied amaryllid genus, *Narcissus* (Baker et al., 2000; Barrett et al., 2004; Cesaro et al., 2004; Sage et al., 1999; Simón-Porcar et al., 2015).

Seed dispersal mechanisms

The tribe Haemantheae have characteristics associated with zoochory, with fleshy, brightly coloured fruits (Gautier-Hion et al., 1985). This differs significantly from other Amaryllidaceae species. Species of the tribe Amaryllideae have different seed dispersal mechanisms that are reliant on abiotic mechanisms such as wind or water as well as autochory (Snijman and Linder, 1996). The tribe Cyrtantheae appear to have evolved for seed dispersal via wind, although this has not been studied.

Seeds or mature fruit of vertebrate-mediated seed dispersal often have toxic secondary metabolites (Whitehead et al., 2022). This may reduce fungal growth, deter unwanted frugivores, and extend gut retention time (Beckman, 2017). Larger seeds appear to have a higher toxicity (Beckman, 2017). Seeds of the southern African *Crinum stuhlmannii* subsp. *delagoense* (Amaryllideae: Amaryllidaceae), have recently been shown to have toxic, alkaloid compounds (Moodley et al., 2022). Seed toxicity is likely prevalent in other amaryllids, such as those studied here.

Kiepiel and Johnson (2019) described a system of monkey seed dispersal for the genus *Clivia* in the tribe Haemantheae. I found the seeds of *Scadoxus multiflorus* subspecies *katherinae* and *S. puniceus* to also be dispersed by monkeys. For *Haemanthus deformis*, which has softer fruits, the seeds were dispersed by birds and rodents. The seeds of Haemantheae are large and could be toxic, making handling difficult. The seeds of Haemantheae are also recalcitrant, germinating soon after the seed has been depulped. For all studies, as well as for *Clivia*, the seed disperser discarded the seeds in some way (monkeys spat out seeds for example).

It appears that habitat is important for determining the animal disperser within Haemanthinae, but this is based on only two studies done here. I have hypothesised that all Haemanthineae, based on fruit morphology, are dispersed by animals but this needs additional research done on different species to be confirmed. For example, very little is known about the fruits and seeds of *Gethyllis* and *Apodolirion*, with speculations including the role of tortoises (Liltved, 1992).

The importance of natural history data and future research

Critically understanding and evaluating plant-pollinator (or -seed disperser) interactions has led to a better understanding of the evolution of plants (van der Niet, 2021). The use of both natural history studies and phylogenetic analysis has given us insights into the role that pollinators play in the evolution of flowering plants (van der Niet and Johnson, 2012; Weller and Sakai, 1999). Work on a single population or species gives a limited understanding of macroevolution and larger scale pollination studies have provided more insight into the evolution of the diversity of flowering plants (Grant and Grant, 1948; Ollerton et al., 2019). The interdisciplinary approach of character mapping on a phylogeny is key in understanding divergent evolution within a group associated with the characters, which may be pollination system or floral morphology associated with pollination system (Weller and Sakai, 1999).

In this thesis, I show how mutualisms with pollinators or seed dispersers are associated with flower and fruit characteristics in the amaryllid subtribe Haemanthinae. Using the recent *Scadoxus* phylogeny of Bødker (2020), I have looked at how shifts in pollinators have potentially resulted in speciation within the genus. To truly understand the potential pollinator shifts within the genus, more work needs to be done to determine the pollinators of other species as well as a more resolved phylogeny. This includes, but is not limited to, the variation found in *S. multiflorus* and *S. puniceus*, which may represent multiple different species instead of one. Although hypotheses are made in this discussion regarding the pollination of other *Scadoxus* species, little research has been done, besides the work reported in this thesis. A notable exception is Mertens et al. (2020) who found butterfly pollination for *S. cinnabarinus*. An understanding of the potential shifts in the group would require formal character reconstruction (Smith, 2010). Without ancestral reconstruction, it is difficult to assume the cause of potential diversifications and whether the consequences of evolution are in fact trait-based (Dodd et al., 1999).

The pollination system of several *Haemanthus* species was investigated and all found to involve sunbirds. It was evident that *H. humilis* subsp. *hirsutus* could be equally reliant on smaller visitors for pollination, although quantification of visits and effectiveness of visits of smaller visitors was not done, leaving many unanswered questions with regards to the pollination of this species. Based on morphology and one small study (Summerfield and van der Walt, 1992), it is likely that other *Haemanthus* species are reliant on insect visitors. In general, there needs to be more natural history data for Haemanthinae, as it is too premature to study shifts in these genera.

A full phylogeny of the Haemantheae is needed to map out characters to make inferences about the role that pollinator shifts may have had in the evolution of *Scadoxus* and *Haemanthus* and other genera in the clade. Because the use of syndromes alone can lead to incorrect conclusions about the history of pollination systems (van der Niet, 2021), I have avoided drawing firm conclusions for this thesis based on floral syndromes alone. Natural history studies therefore remain critical for understanding plant evolution (van der Niet, 2021) and further natural history research into the Haemantheae may provide not only more understanding into this group, but also may provide answers with regards to understanding pollinator shifts as a whole by using the Haemantheae as a key example of pollinator shifts within a larger group of flowering plants.

The use of camera traps proved indispensable throughout the work reported here. This elucidated visitors more easily than hours of direct observations and provided good data on pollinator activity that perhaps may have not been seen via direct observations. Camera traps can therefore be used solve the missing natural history problem as they are a reliable mechanism to observe pollinator and seed disperser behaviour (Krauss et al., 2018; Ortmann and Johnson, 2020). This method could be used to discover the pollination and seed dispersal systems of other *Haemanthus* and *Scadoxus* species and provide a better understanding of the group.

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CORRIGENDUM FOR PUBLISHED PAPERS

Chapter 3

Under the methods section under the subheading controlled pollination: Cross pollinations were done using tweezers to remove anthers from a separate individual whose pollen was then dusted onto the stigma.

Discussion: Pollination efficiency may be directly affected by the number of honeybees seen to visit *Scadoxus puniceus*. Honeybees are widespread in south Africa and are known to be pollen thieves and ineffective pollinators of bird-adapted flowering species (Hargreaves et al., 2012).

Chapter 5

Sample sizes for Figure 2: *Scadoxus multiflorus* subspecies *katherinae*: 11 bouts; *S. puniceus*: 14 bouts.

Sample sizes for Figure 3: *Scadoxus multiflorus* subspecies *katherinae*: 10 plants; *S. puniceus* at VC: 23 plants; at KF: 25 plants.

Discussion: Vervet monkeys (*Cercopithecus aethiops*) are known to have a broad diet, including many fruit species. It is likely that *Scadoxus* fruits form a small portion of such this. Other than the fruits of *Clivia miniata*, which are large with large seeds and are dispersed by monkeys (Kiepiel and Johnson, 2019), the majority of fruits possibly consumed by monkeys are relatively small.

Discussion: During the study, nothing was seen to predate or interact with the seeds of Scadoxus. We assume that this may be due to the seeds' possible toxicity (Nair and van Staden, 2022).