The reproductive ecology of *Aloe reitzii* var. *reitzii* (Asphodelaceae): a South African grassland endemic

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DECLARATION

I declare that this dissertation is my own, unaided work. It has been submitted in fulfilment of the requirements of a Master of Science at the University of the Witwatersrand. It has not been submitted before for any degree or examination to another university or similar institution.

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ABSTRACT

According to the pollination syndrome hypothesis, floral traits associated with attracting particular pollinators are used to infer likely pollinators. Most *Aloe* species are characterized by a bird-pollination (ornithophilous) syndrome, as seen by their red/orange floral displays and copious nectar production. Indeed, many aloe species are primarily bird-pollinated, but small non-flying mammals and insects, particularly bees, are also effective pollinators. Additionally, aloes have been shown to host a variety of phytophages and their associated parasatoids, but little is known about the effect these parasites have on the plants' reproductive output. *Aloe reitzii* var. *reitzii* is a summer-flowering aloe, endemic to the grasslands in the eastern regions of South Africa; it is abundantly visited by birds and bees. However, the roles of these two pollinator groups in the pollination of *A. reitzii* var. *reitzii* have not been assessed. This study aimed to investigate the breeding system of *A. reitzii* var. *reitzii* and the relative roles of birds and insects in its reproductive biology – both in pollination and fruit and seed predation. During the 2020 flowering season, observations of floral visitors were conducted to identify the main floral visitors to *A. reitzii* var. *reitzii*, and their visitation rates. The role of these floral visitors in the pollination of *A. reitzii* var. *reitzii* was determined by swabbing birds and insects for pollen, and their relative contributions to the reproductive output of *A. reitzii* var. *reitzii* was determined using pollinator exclusion experiments. Since generalist and specialist nectarivorous birds are common visitors to *A. reitzii* var. *reitzii*, their roles in its pollination were assessed by i) measuring nectar properties, ii) comparing main sites of pollen deposition, and iii) comparing their bill morphology to floral characteristics of *A. reitzii* var. *reitzii.*

Contrary to most *Aloe* species, *Aloe reitzii* var. *reitzii* is self-compatible, a condition previously reported in only one other South African *Aloe* species. However, *A. reitzii* var. *reitzii* is not capable of autonomous self-pollination, requiring pollinators to transfer pollen between flowers within a raceme and between racemes on a plant. Bees are shown to facilitate self-pollination, suggesting a mixed-mating system in *A. reitzii* var. *reitzii*. *Aloe reitzii* var. *reitzii* is predominantly bird-pollinated, which is consistent with its floral traits and nectar properties. Pollinator exclusion experiments show birds are the predominant pollinator in *A. reitzii* var. *reitzii*, and bees only play a minor role. The long floral tubes and generalist nectar properties of *A. reitzii* var. *reitzii* suggest floral traits are an intermediate between a generalist and specialist pollination syndrome, enabling effective pollination by both generalist and specialist nectarivores, Cape Weavers (*Ploceus capensis*) and Malachite Sunbirds (*Nectarinia famosa*), respectively. Although pollen loads did not differ between these two species, due to their consistent abundance and frequent visitation at its flowers over multiple years, *A. reitzii* var. *reitzii* is primarily pollinated by Cape Weavers. *Aloe reitzii* var. *reitzii* is host to a variety of phytophages and parasitoids, with the chalcid wasp *Euryoma aloineae* as the predominant pre-dispersal seed predator in *A. reitzii* var. *reitzii*. Fruit and seed predation by phytophagous and parasitoid insects greatly reduces the reproductive output of *A. reitzii* var. *reitzii*, which may considerably impact the persistence of its populations, especially its fragmented populations.

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LIST OF ABBREVIATIONS

a.s.l.	Above sea level
CV	Coefficient of Variation
GLMM	Generalized Linear Mixed Effects Model
RGC	Room Germination Conditions
SGC	Standard Germination Conditions

CHAPTER ONE

General Introduction

Rationale

According to the pollination syndrome hypothesis, diverse floral traits are suggested to reflect pollination syndromes of animal-pollinated plants (Fenster *et al*., 2004; Ollerton *et al*., 2009). As a result, floral characteristics such as flowering period, floral scent and nectar, flower colour, size and morphology (Fægri and van der Pijl, 1979; Ollerton *et al*., 2009) have been used to predict likely pollinators of plants, particularly in the absence of direct observations (Ollerton *et al*., 2009). Pollination syndromes therefore suggest angiosperms are specialized for pollination by particular animal types and reflect convergent syndromes of floral traits (Johnson and Steiner, 2000; Ollerton *et al*., 2009). Since pollination syndromes have been used to organize the phenotypic diversity of flowers, they provide a mechanistic explanation for floral diversity and infer pollinators in the absence of direct observations. Pollination syndromes have therefore played a central role in studies of pollination biology (Ollerton *et al*., 2009). Even though observations of convergent floral morphological traits have been successfully used to generate hypotheses about the ecology of pollination systems (Rosas-Guerro *et al*., 2014), it has also been shown that they do not successfully describe floral phenotypic diversity or predict the pollinators of many plant species (Ollerton *et al*., 2009).

Aloes are amongst the most threatened groups of plants in Africa, many of which have been placed under CITES protection (Kumar, 2006). Various *Aloe* L. species are exploited for their medicinal properties and used in multiple natural products in the health and cosmetic industries (e.g., *Aloe vera* (L.) Burm.f.; Grace *et al*., 2009; Cousins and Witkowski, 2012), but wild populations are also under threat due to habitat loss and large-scale collection from the wild for horticulture (Cousins and Witkowski, 2012). In addition, the decline of many *Aloe* species can be attributed to poor farming practices and trampling by farm animals, afforestation, mining and urbanization (Cousins and Witkowski, 2012).

The genus *Aloe* comprises c. 580 species, most of which are found in Africa, concentrated in southern and eastern Africa, as well as in Madagascar (Cousins and Witkowski, 2012; Govaerts and Newton, 2019). South Africa is the major centre of aloe diversity, with c. 140

taxa — the largest number of aloes of any African country, most of which (c. 80 species) are found in the northern provinces of the country (Reynolds, 1969; Van Jaarsveld, 1989; Cousins and Witkowski, 2012). The pollination biology and breeding system of many aloes has been well-investigated (Johnson *et al.*, 2006; Botes *et* al., 2009a,b; Hargreaves *et al.*, 2012; Patrick *et al.*, 2018), but the breeding systems of some aloes have not been experimentally investigated (e.g., *Kumara plicatilis* (L.) G.D.Rowley, Cousins *et al.*, 2013; *A. peglerae* Schonl., Arena *et al.*, 2013). Investigating the breeding systems and pollinator contributions to the reproductive output of aloes, and their impact on subsequent generations, is important in determining the diversity and success of plant-pollinator interactions within the genus (Arena *et al.*, 2013). Thus, further studies on the reproductive ecology of *Aloe* species will contribute to the management and conservation of wild aloe populations and their respective habitats (Cousins and Witkowski, 2012).

The floral features of many South African aloes are consistent with an ornithophilous (bird) pollination syndrome (Cousins and Witkowski, 2012), characterized by their red-orange tubular perianth, exserted stamens, high production of nectar, and the absence of any odour and nectar guides (Hoffman, 1988; Stokes and Yeaton, 1995; Symes and Nicolson, 2008a; Symes *et al.*, 2008). Even though birds play a primary role in the pollination system of many aloes (Johnson *et al.*, 2006; Symes and Nicolson, 2008b; Botes *et al.*, 2009a; Hargreaves *et al.*, 2012; Arena *et al.*, 2013; Appendix A), insects, particularly bees, are also shown to be effective pollinators of aloes (e.g., Hoffman, 1988; Hargreaves *et al.*, 2008; Botes *et al.*, 2009a,b; Symes *et al.*, 2009; Shibanda, 2016; Patrick *et al.*, 2018). However, the contribution of insects, particularly honeybees, in the pollination of ‘ornithophilous’ aloes, is not well studied (Botes *et al.*, 2009a). Hence, future studies need to include quantification of the role bees play in reproductive ecology to determine the general importance of bees in aloe pollination ecology, especially in aloes apparently adapted for bird pollination.

Aloe reitzii Reynolds var. *reitzii* (from here-on referred to as *Aloe reitzii*) is a summer-flowering aloe (Van Wyk and Smith, 2008), endemic to the grasslands of northern South Africa. *Aloe reitzii* has an “ornithophilous” pollination syndrome, with birds observed as regular visitors to this species, thus they likely play an important role in its pollination (Symes, 2017; Payne, 2019). However, insects are also common visitors (Symes, 2017; Payne, 2019), but the role of insects in the pollination system of *A. reitzii* has not been confirmed nor quantified. Generalist and specialist nectarivore bird species are both frequent visitors to *A.*

reitzii flowers (Symes, 2017; Payne, 2019), but their relative importance to the pollination of *A. reitzii* has not been investigated. Therefore, the relative roles that both birds and insects play in the pollination system of *A. reitzii* needs to be quantified, as well as whether *A. reitzii* is dependent on these floral visitors for reproduction and seed set.

Literature Review

Pollination Syndromes

Most angiosperms are pollinated by biotic factors (i.e., animals), rather than by abiotic factors, such as wind or water (Waser, 2006; Ollerton *et al*., 2011). Plant-pollinator interactions form a key ecological process, as plants are dependent on their pollinators for pollen transfer and seed set, and plants usually provide a reward for animal pollinators, in the form of pollen, nectar and other rewards (Kearns *et al.*, 1998), such as oils and resins (Fægri and van der Pijl, 1979). In addition to floral rewards as primary attractants for pollinators, floral traits such as colour, odour and floral structure (shape and size of flowers) are also associated with attracting particular pollinators (Fægri and van der Pijl, 1979). Pollinator-mediated selection on floral traits has therefore been considered an important evolutionary force underlying the diversification of flowering plants (Pauw, 2006; Rosas-Guerro *et al.*, 2014).

A pollination syndrome is defined as a “suite of floral traits, including rewards, associated with the attraction and utilization of a specific group of pollinators” (Fenster *et al.*, 2004, p. 376). The term implies that pollinators can be grouped into specific functional groups that behave similarly and exert similar selection pressures on plants, resulting in the observed specialization for different groups of pollinators (Fenster *et al.*, 2004). Selection on floral traits by pollinator functional groups is said to result in convergent floral traits associated with specific pollinators (Fenster *et al.*, 2004). Hence, flowering season and floral traits (e.g., morphology, colour, scent, size, and rewards) signify the most effective and/or abundant pollinator through specialization of these traits, reflecting foraging preferences and morphology of their pollinators (Fægri and van der Pijl, 1979). Therefore, the role of pollinators as selective agents of floral diversity and the maintenance of floral trait combinations can be quantified (Armbuster *et al.*, 2000). The pollination syndrome hypothesis therefore implies that specialization, where plants are only pollinated by a small subset of pollinator functional groups (Armbuster, 2012), commonly occurs (Fenster *et al.*, 2004). However, functional groups differ in their effectiveness as pollinators; thus a plant may be visited by several functional groups, but each will exert different selective pressures on the floral traits (Fenster *et al.*, 2004).

According to Stebbins' (1970) "most effective pollinator principle", pollinators that are the most frequent visitors and the most effective pollinators usually exert the strongest selective pressures on floral traits. Therefore, diverse floral structures and pollination mechanisms in angiosperms are a result of adaptive radiations in response to different pollen vectors and different ways of adapting to the same vector (Stebbins, 1970). Stebbins' (1970) principle suggests natural selection modifies plant phenotypes due to plant-pollinator interactions (Gómez and Zamora, 2006), and similar to Fenster *et al.*'s (2004) definition of pollination syndromes, plants are expected to be predominantly visited by a small group of highly effective pollinators (adaptive specialization; Herrera, 1996; Gómez and Zamora, 2006). However, for natural selection to occur, the relative importance of various floral visitors as pollinators not only depends on the frequency of their visits (and abundance at flowers), but also depends on their ability to transfer pollen successfully between flowers of the same species (per-visit effectiveness; Gómez and Zamora, 2006). Therefore, just because a flower may have floral traits indicative of the predominant and most effective floral visitor, it does not mean the plant is exclusively pollinated by this visitor (Stebbins, 1970).

In addition to being used to explain the convergent adaptation for specific types of pollinators, pollination syndromes are commonly used to organize the high phenotypic diversity of flowers, and to infer potential pollinators in the absence of direct observations (Ollerton *et al.*, 2009). Consequently, pollination syndromes have been central in the development of pollination biology (Ollerton *et al.*, 2009). However, even though there may be a strong association between floral traits and functional groups of pollinators (Fenster *et al.*, 2004), it is often difficult to distinguish which animals serve as pollinators or just visitors (Fægri and van der Pijl, 1979). Therefore, despite the success of the "pollination syndrome hypothesis", the reliability of pollination syndromes in predicting primary pollinators and the assumption of specialization in pollination systems has been questioned (Waser *et al.*, 1996; De Witt Smith *et al.*, 2008; Ollerton *et al.*, 2009).

To assess the validity of the "pollination syndrome hypothesis" in predicting primary pollination and specialization, many comparative studies have investigated the relationship between plants and their pollen vectors, often resulting in conflicting results. Some studies suggest more plants display moderate generalized syndromes compared to the 'rule' of specialization (e.g., Ollerton, 1996; Waser *et al.*, 1996; De Witt Smith *et al.*, 2008; Ollerton *et al.*, 2009), whereas other studies of the same plants (Carlinville flora, USA) as Waser *et al.* (1996), and their southern hemisphere counterparts, were shown to be specialized into functional groups (Johnson and Steiner, 2000; Fenster *et al.*, 2004). Additionally, a quantitative review of pollination syndromes revealed that floral syndromes can successfully predict the most effective pollinator, regardless of breeding system, geographic distribution and pollinator functional group (Rosas-Guerro *et al.*, 2014). Despite the success of the pollination syndrome hypothesis, the majority of studies have focused on a single plant species or taxonomic group (e.g., Johnson and Steiner, 2000; Fenster *et al.*, 2004). Additionally, Stebbins' (1970) 'most effective pollinator principle' does not take into account the complexity of plant-pollinator interactions and the wide range of possibilities of plant-pollinator relationships (Ollerton *et al.*, 2009; Rosas-Guerro *et al.*, 2014).

Secondary pollinators may also play a role in plant reproduction, acting as antagonists or enabling reproduction when primary pollinators are scarce, thus also potentially causing evolutionary shifts that may impact pollination syndromes (Rosas-Guerro *et al.*, 2014). Plants may therefore reflect floral traits of 'major' pollinators but can be effectively pollinated by 'minor' visitors (Aigner, 2001). For example, *Erica halicacaba* L. (Ericaceae) has floral traits indicative of insect pollination (viz., short-tubed, green-yellow flowers) but was found to be bird-pollinated (Turner *et al.*, 2012); similarly, plants with floral traits suggesting bird- or rodent-pollination are also effectively pollinated by insects (e.g., *Protea caffra* Meisn.; Steenhuisen *et al.*, 2012). This implies that one cannot simply infer agents of floral adaptations solely based on pollination syndromes (Ollerton *et al.*, 2007), and pollination syndromes cannot always be accepted as evidence that a plant is pollinated by certain animals (Hargreaves *et al.*, 2004). Therefore, caution should be taken when using the pollination syndrome hypothesis to predict primary/most effective pollinators (Ollerton *et al.*, 2009). However, it should be noted that much of the conflict around the use of pollination syndromes has resulted from the view that pollination syndromes reflect the coevolution of plant and pollinator species (which is rare), and failure to distinguish between evolutionary and ecological specialization (Armbuster *et al.*, 2000).

The distinction between evolutionary specialization and ecological specialization is crucial to understanding pollination ecology (Armbuster *et al.*, 2000), as a fundamental aspect of pollination mutualism is the degree to which plants are specialized (Armbuster, 2012). In regard to pollination, evolutionary specialization refers to a change from pollination by many animal species to pollination by fewer species, whereas ecological specialization refers to only having a few pollinators of similar animal species at the present time (Armbuster *et al.*, 2000). Ecological generalization, on the other hand, is when flowers are visited by many species of a variety of functional groups of animals (Fenster *et al.*, 2004). Compared to generalized pollination systems, specialized pollination systems are characterized by specific odours and colours, floral morphology that may restrict access by specific animals to nectar, or in some plants, to specialized rewards such as oils (e.g., *Diascia* Link & Otto; Steiner and Whitehead, 1991a) or resins (e.g., Euphorbiaceae; Steiner and Whitehead, 1991b). Even though specialized rewards or floral morphology may decrease the diversity of floral visitors, it rarely leads to absolute specialization (pollination by only one species; Armbuster *et al.*, 2000) of the flower to these visitors (Minckley and Roulston, 2008). Thus, dividing pollination systems into specialization and generalization is an over-simplification of the continuum between plants pollinated by many animal species and plants pollinated by just one species (Johnson and Steiner, 2000).

Plant-pollinator interactions range from obligate specialists (a single pollinator species pollinates one plant species) to facultative generalists, in which flowers may be pollinated by any number of a taxonomically broad range of pollinators, which are also visitors to other species (Ollerton *et al.*, 2007). Most angiosperms follow a generalist pollination system, which may include pollinators that vary across orders of insects (e.g., butterflies, bees, flies) and may often include different classes of animals (e.g., birds and mammals; Ollerton, 1996). However, despite the vast majority of visitors pollinating flowers, not all pollinators equally pollinate flowers, thus effectively making the plant a specialist (Ollerton, 1996). Therefore, specialization and generalization should be considered as a continuum across different biological scales, each with different functional implications (Brosi, 2016).

The relationship between flower structure and pollen vectors can develop along a range of evolutionary pathways, involving both adaptation to different pollen vectors and different ways of adapting to the same pollen vector (Stebbins, 1970). Significant factors that influence the relationship between pollen vectors and their plants are the frequency of a particular vector, limitations imposed by the existing structure of the flower, and limitations imposed by the external environment (Stebbins, 1970). Frequency of visits to the flower by pollen vectors is influenced by means of one or more direct attractants, namely nectar, pollen and scent (Fægri and van der Pijl, 1979), with nectar and pollen serving as the main food sources for floral visitors (Proctor *et al.*, 1996). Nectar is therefore the most common reward attracting pollinators, with nectar traits such as sugar concentration and nectar volume impacting the types of floral visitors (Armbuster, 2012).

Ornithophilous pollination syndrome

Although insects are the most important animal pollinators, vertebrates, especially birds, play a significant role in the pollination of angiosperms (Proctor *et al.*, 1996). Evolutionary shifts to bird pollination, i.e., ornithophily, have occurred independently in many lineages of flowering plants (e.g., *Ipomoea* L. and *Mimulus* L.; Cronk and Ojeda, 2008). A shift to ornithophily has therefore resulted in morphological changes in many floral features within lineages, particularly those associated with the attraction of birds, deterrence of ineffective floral visitors, protection from foraging by nectar-thieving birds, and accurate placement of pollen on birds' bodies (Proctor *et al.*, 1996; Cronk and Ojeda, 2008; Pauw, 2019). Pollination by nectarivorous bird species plays an important role in the ecosystem function for thousands of plant species worldwide (Abrahamczyk, 2019).

Many plant families are bird-pollinated; thus, the basic structure of bird-pollinated flowers is diverse (Proctor *et al.*, 1996). However, ornithophily is mainly characterized by a broad syndrome of convergent traits, namely, a tubular perianth, red or orange colour, odourless flowers (Fægri and van der Pijl, 1979; Ollerton, 1998; Pauw, 2019), and abundant dilute nectar (Fægri and van der Pijl, 1979; Stiles, 1981; Proctor *et al.*, 1996; Ollerton, 1998; Cronk and Ojeda, 2008; Pauw, 2019), which serves as the main floral reward for birds, and is suggested to be the main attractant to birds (Stiles, 1976).

Compared to insects, the large size and homeothermy of birds increases their energy requirements, whereas insects reduce their metabolism in response to unfavourable conditions (Stiles, 1978). Therefore, birds are more reliable pollinators, especially during unpredictable climates and flowering seasons, and during winter when many bird-pollinated plants flower (Fægri and van der Pijl, 1979; Geerts *et al.*, 2020), as birds must continue foraging when many insects remain inactive (Stiles, 1978). During the winter months, many South African plant species provide a vital food source and meet the high energy demands of birds through the production of copious amounts of nectar (Hoffman, 1988; Hargreaves *et al.*, 2004; Symes *et* *al*., 2008; Botes *et al*., 2009a,b; Brown *et al*., 2009, 2010; Geerts *et al*., 2020). The structure and composition of avian communities change spatially and temporally with the availability of resources, and plants, such as aloes, are shown to drive seasonal changes in avian communities (e.g., *Aloe marlothii* A.Berger, Symes *et al*., 2008; *Aloe ferox* Mill., Forbes *et al*., 2009; Kuiper *et al*., 2015). As a result, birds are more constant visitors compared to insects in winter (Stiles, 1978).

Because of the large size and high energy requirements of birds, ornithophily is energetically expensive for plants and should only occur when birds can maximize pollen flow and seed set (Stiles, 1978). Nectar is the only floral reward offered to birds and serves as an important energy source for many birds (Stiles, 1978; Fægri and van der Pijl, 1979; Stiles, 1981; Cronk and Ojeda, 2008). Therefore, to meet the high energy requirements of birds, flowers of bird-pollinated plants secrete large quantities of nectar, which is relatively dilute (Proctor *et al*., 1996), often found dripping out of flowers, e.g., *Protea repens* (L.) L (Geerts and Pauw, 2011) and *Aloe marlothii* (Symes and Nicolson, 2008a). Compared to insect-pollinated flowers, bird-pollinated flowers are characterized by high nectar volumes, and lower sugar concentrations, reflecting the high energy requirements of birds (Stiles, 1978; 1981). Therefore, other insects, such as bees, will be attracted to bird-pollinated plants with such a high nectar reward, sometimes removing nectar and pollen without effecting pollination (Hargeaves *et al*., 2010).

Bird-adapted flowers are conspicuous, characterized by vivid floral displays (red coloration and large flowers; Proctor *et al*., 1996; Cronk and Ojeda, 2008). Bird visual cues are sensitive to red colours (Proctor *et al*., 1996; Fægri and van der Pijl, 1979), which lie in the long-wavelength end of the visible spectrum (Stiles, 1976). However, birds have also been shown to be effective pollinators of flowers with colours such as orange and yellow [e.g., *Satyrium coriifolium* Sw. (Orchidaceae), Johnson, 1996] and white [e.g., *Angraecum striatum* Thouars (Orchidaceae), Micheneau *et al*., 2006]. However, a study on Anna Hummingbirds (*Calypte anna*), in California, showed that long-wavelength-coloured flowers (viz., orange and magenta) are highly preferred by birds, whereas short-wavelength-coloured flowers (e.g., pink, white and yellow) are weakly preferred or not preferred at all (Stiles, 1976). Additionally, red coloration also serves as an indicator of high caloric reward for birds (Raven, 1972). Hence, red coloration of flowers may serve as an orienting stimulus, perceived from a distance that directs birds to nectar, especially in shady or very open, sunlit habitats, where red colours are more conspicuous to birds compared to green/dull backgrounds (Stiles, 1976).

The genus *Aloe*

South Africa has the largest number of *Aloe* species of any African country (140 taxa), most of which (c. 80 species) occur in the northern provinces of the country (Reynolds, 1969; van Jaarsveld, 1989; Cousins and Witkowski, 2012). Most South African *Aloe* species flower in winter, with c. 40% of species flowering in summer (Van Wyk and Smith, 2008). The pollination biology and breeding system of aloes has been well-investigated, e.g., *A. ferox* (Hoffman, 1988; Stokes and Yeaton, 1995; Botes *et al.*, 2008, 2009a; Hargreaves *et al.*, 2012), *A. marlothii* (Symes and Nicolson, 2008b; Hargreaves *et al.*, 2012) and *A. greatheadii* var. *davyana* (Schönland) Glen & D.S.Hardy (Symes *et al.*, 2009; Hargreaves *et al.*, 2012). Although the pollination biology of some aloes has been studied, the breeding system of these aloes has not yet been experimentally investigated, such as *A. peglerae* (Arena *et al.*, 2013; Payne *et al.*, 2016) and *Kumara plicatilis* (Cousins *et al.*, 2013).

Aloe pollination

The majority of *Aloe* species studied have been shown to be self-incompatible, requiring cross-pollination by pollen vectors for reproduction and seed set (Appendix A, Johnson *et al.*, 2006; Hargreaves *et al.*, 2008; Botes *et al.*, 2009a,b; Symes *et al.*, 2009; Wilson *et al.*, 2009; Hargreaves *et al.*, 2012). However, autonomous self-pollination was observed in *A. maculata* Medic. and *A. kraussii* Baker (Hargreaves *et al.*, 2012), as well as in *A. peglerae* (Arena *et al.*, 2013), but seed set was low in all three species (Hargreaves *et al.*, 2012; Arena *et al.*, 2013). Some aloes are self-compatible and capable of uniparental production, such as *A. thraskii* Baker (Patrick *et al.*, 2018) and *A. divaricata* Berger (Madagascar; Ratsirarson, 1995). However, *A. thraskii* and *A. divaricata* still require a vector for the movement of pollen between flowers within a plant and between neighbouring plants (Ratsirarson, 1995; Patrick *et al.*, 2018).

Most *Aloe* species have floral traits characteristic of an “ornithophilous” pollination syndrome, namely their red/orange colour, a long tubular perianth, exserted stamens, copious dilute nectar, and an absence of nectar guides and odour (Hoffman, 1988; Johnson *et al.*, 2006; Botes *et al.*, 2009a; Wilson *et al.*, 2009; Hargreaves *et al.*, 2012; Patrick *et al.*, 2018). The high quantities of nectar serve as a vital food source for both birds and insects, especially during the dry winter months when other food sources are scarce (Symes *et al.*, 2008; Cousins and Witkowski, 2012). Therefore, consistent with their ornithophilous floral traits, birds play a primary role in the pollination system of many South African aloes (Stokes and Yeaton, 1995; Botes *et al.*, 2009b; Arena *et al.*, 2013), with most *Aloe* species adapted for specialist sunbird pollination (Cousins and Witkowski, 2012; Appendix A). However, generalist nectarivores are also primary pollinators of many *Aloe* species (e.g., *A. vryheidensis* Groenew., Johnson *et al.*, 2006; *A. marlothii*, Symes and Nicolson, 2008b; and *A. peglerae*, Arena *et al.*, 2013, Payne *et al.*, 2016). For example, Cape Weavers are primary pollinators of *A. marlothii* (Symes and Nicolson, 2008b), Cape Rock-thrushes pollinate *A. peglerae* (Arena *et al.*, 2013), and a range of short-billed generalists (e.g., Dark-Capped Bulbuls, Cape White-eyes, Streaky-headed Canaries etc.), are effective pollinators of *A. vryheidensis* (Johnson *et al.*, 2006).

At least two distinct bird pollination systems have been recognized in ornithophilous *Aloe* species, with floral traits and nectar properties separating specialist (true) and generalist (opportunistic) nectarivore bird species (Botes *et al.*, 2009a). For example, aloes with specialist sunbirds as pollinators are mainly characterized by mid-length to long-tubed flowers and produce small volumes of relatively concentrated nectar (e.g., *Aloe arborescens* Mill. and *A. maculata*), whereas aloes pollinated by shorter-billed generalist bird species have relatively short-tubed flowers with large volumes of relatively dilute nectar (e.g., *Aloe marlothii* and *A. vryheidensis*, Botes *et al.*, 2009a; Hargreaves *et al.*, 2012; and *A. ferox*, Botes *et al.*, 2008, 2009a; Kuiper *et al.*, 2015; Diller *et al.*, 2019; Appendix A). However, a recent study on *A. kraussii* indicated that long-billed sunbirds can be effective pollinators of short-tubed *Aloe* flowers (Hargreaves *et al.*, 2019). The copious nectar produced by aloes, along with the strongly exserted stamens and abundance of pollen, also makes aloes attractive to insects (Botes *et al.*, 2009a). Therefore, in addition to birds, most *Aloe* species are visited by a wide variety of insects, particularly nectar- and pollen-collecting bees (Hoffman, 1988; Stokes and Yeaton, 1995; Hargreaves *et al.*, 2008; Botes *et al.*, 2009a; Symes *et al.*, 2009; Arena *et al.*, 2013; Duffy *et al.*, 2014).

Both solitary bees (e.g., *Lasioglossum* spp. and *Amegilla* spp.) and colonial bees (e.g., allodapine bee species) are known to be associated with aloes (Hoffman, 1988; Botes *et al.*, 2009b; Patrick *et al.*, 2018). However, African honeybees (*Apis mellifera*, a colonial species) are the predominant floral visitors for many *Aloe* species (e.g., *A. ferox*, Hoffman, 1988; *A.*

minima Baker. and *A. linearifolia* A.Berger, Botes *et al.*, 2009b; *A. thraskii,* Patrick *et al.*, 2018; *A. reitzii* var. *reitzii,* Payne, 2019; Appendix A). Bees play a varying role in the pollination of many aloes, either as primary pollinators (e.g., *A. greatheadii* var. *davyana,* Symes *et al.*, 2009; *A. inconspicua* Plowes, Hargreaves *et al.*, 2008; *A. linearifolia* and *A. minima*; Botes *et al.*, 2009b), co-pollinators (e.g., *A. arborescens,* Hargreaves *et al.*, 2012; *A. boylei* Baker, Hargreaves *et al.*, 2012; *A. lineata* var. *muirii*, Botes *et al.*, 2009a), or negatively as pollen thieves (e.g., *A. maculata*, Hargreaves *et al.*, 2010). Bees are abundant floral visitors to *A. reitzii* (Symes, 2017; Payne, 2019), but their contribution to *A. reitzii* pollination has not yet been investigated.

Parasitism/predation in Aloe

Insects have been shown to play a role in the pollination of aloes, either as primary pollinators (Botes *et al.*, 2009b; Symes *et al.*, 2009; Hargreaves *et al.*, 2012; Cousins *et al.*, 2013) or co-pollinators or secondary pollinators (Hoffman, 1988; Botes *et al*., 2009a,b; Patrick *et al*., 2018), or as pollen thieves (Hargreaves *et al.*, 2010), but little is known about the role of insects as predators, and their effect on the reproductive output of aloes. A recent study on *Aloe pretoriensis* Pole Evans showed that its fruit and seeds are host to a variety of phytophages and their associated parasitoids, including six wasp species (e.g., *Eurytoma aloineae* (Chalcididae), *Mesopolobus* sp. and *Pteromalus* sp.) and a fruitfly (*Apenthecia* sp.; van den Bosch *et al*., 2019). This predation and parasitism resulted in considerable reduction in the fruit set of *A. pretoriensis* (48 – 93%) and seed set (7 – 68%; van den Bosch *et al*., 2019). Insect-related fruit and seed predation, especially by *Eurytoma* sp. wasps, is also known to reduce the quantity of intact seeds dispersed from fruit of *A. lettyae* Reynolds (Kremer-Köhne *et al.*, 2020). Hence, predation/parasitism can greatly reduce the reproductive output of aloes, which may drastically impact aloe populations. The presence of shriveled fruit was observed on *A. reitzii* (on 33% of plants; Payne, 2019). However, it is unknown whether this could be due to insect predation or the presence of fungus or bacteria (disease). Therefore, it is important to investigate the role of predation by insects, particularly small phytophagous and/or parasitoid wasps, on the reproductive output of aloes.

Aloe reitzii *var.* reitzii

Aloe reitzii is a grassland aloe endemic to South Africa, comprising two varieties, *Aloe reitzii* var. *reitzii* and *A. reitzii* Reynolds var. *vernalis* D.S.Hardy, which differ in morphology, flowering season and distribution. *Aloe reitzii* var. *reitzii* (Figure 1A) is a summer-flowering aloe distributed in Mpumalanga, flowering between February and March, with localized distributions in Limpopo and Gauteng (Van Wyk and Smith, 2008). *Aloe reitzii* var. *vernalis*, on the other hand, flowers in spring in August and September, and has a very restricted distribution in northern KwaZulu-Natal (Van Wyk and Smith, 2008). The smaller bracts and smaller fruits of *A. reitzii* var. *vernalis* distinguish it from *A. reitzii* var. *reitzii* (Van Wyk and Smith, 2008). *Aloe reitzii* var. r*eitzii* is Near-Threatened for reasons outlined below (Mtshali *et al*., 2018a), and due to its restricted distribution, *Aloe reitzii* var. *vernalis* is Vulnerable (Mtshali *et al*., 2018b).

Populations of *Aloe reitzii* var. *reitzii* (hereafter referred to as *A. reitzii*) are increasingly declining due to habitat loss, primarily caused by commercial timber plantations, mining and quarrying development, with at least 30% of their habitat irreversibly modified (Mtshali *et al.*, 2018a). Additionally, farming practices in the area may further cause population decline. As with most South African aloes, *A. reitzii* has an ornithophilous pollination syndrome, characterized by its long (~30 – 50 mm), narrow, red-orange tubular flowers (Van Wyk and Smith, 2008; Figure 1B) and copious nectar production (Symes, 2017; Payne, 2019).

Aloe reitzii is visited by both birds and insects; however, birds are suggested to play a more significant role in its pollination system (Symes, 2017; Payne, 2019). Even though the floral structure and nectar properties of *A. reitzii* suggest specialist nectarivorous bird pollination, both specialist (e.g., Malachite Sunbird *Nectarinia famosa*) and generalist nectarivores (e.g., Cape Weavers *Ploceus capensis*) were common visitors to an *A. reitzii* population near Dullstroom, Mpumalanga (Symes, 2017; Payne, 2019). Insects were also common visitors (Symes, 2017; Payne, 2019), with honeybees observed drinking nectar and removing pollen (Payne, 2019). However, the role of insects in the pollination of *A. reitzii* has not been confirmed or quantified, but their abundance at the same *A. reitzii* population near Dullstroom in multiple years suggests that they may be important role players in its pollination system (Symes, 2017). Therefore, the relative roles that both birds and insects, particularly bees, play in the pollination system of *A. reitzii* need to be quantified, as well as their role in the reproduction of *A. reitzii*.

In an initial, short-term study (~ three days) on Klipbankspruit Farm, generalist visitors (Cape Weaver) visited *Aloe reitzii* more often, contributing to 60% of all visits, whereas specialist bird species (Malachite Sunbird and the Greater Double-collared Sunbird *Cinnyris* *afer*) contributed substantially to visits (27% and 13% respectively; Symes, 2017). The opposite pattern was observed in a longer study at the same study site as Symes (2017), three years later (~three weeks through the peak flowering season), with Malachite Sunbirds contributing 49% of all visits compared to Cape Weavers (41% of all visits; Payne, 2019). In addition, nectar properties reported by Symes (2017) suggest that *Aloe reitzii* is more likely pollinated by sunbirds (mean volume: 36 µL ± 27 µL; mean concentration: 13.5%–19.5% w/w), whereas Payne (2019) found that nectar properties reflected generalist preferences (volume range: 37 µL – 100 µL; concentration range: 9.1 - 11.5% w/w). Thus, generalist and specialist bird species may both be important pollinators of *A. reitzii.*

In addition to birds, honeybees (*Apis mellifera*) were observed removing nectar (Payne, 2019), thus potentially playing a role in pollination of *A. reitzii*. Other insects, such as grasshoppers and beetles were common floral visitors, but were likely eating floral parts and playing no role in pollination. Additionally, hawkmoths were recorded visiting *A. reitzii* at night, but their role as potential pollinators was not confirmed nor quantified. Honeybees and hawkmoths may therefore be important role players in the pollination of *A. reitzii*, but may be less important pollinators than specialist and/or generalist bird species (Symes, 2017). Therefore, it is important to identify the primary pollinator guild and their role in the pollination of *A. reitzii.*

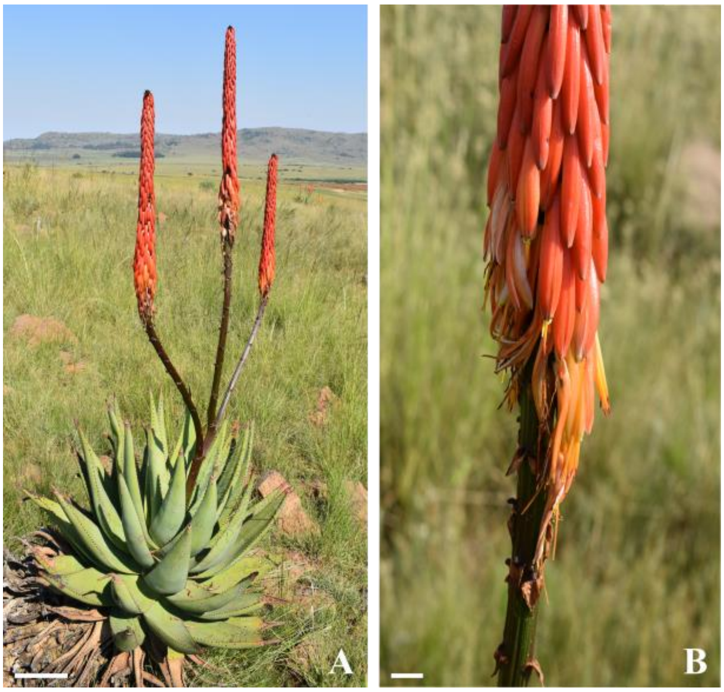

Figure 1**.** Flowering (mature) *Aloe reitzii* var. *reitzii* plant (A), with its raceme of tightly packed, red-orange tubular flowers (B). Scale bars: A = 10 cm, B = 1 cm. Photographs by Jessica Minnaar.

Aims and Objectives

This study aims to investigate i) whether *A. reitzii* depends solely on cross pollination and is reliant on pollen vectors for reproduction, and ii) the relative roles of birds and insects in its reproductive biology – both pollination and predation. The objectives associated with this aim are as follows:

1) To investigate the breeding system of *A. reitzii* to determine whether it is self-incompatible, thus reliant on cross-pollination for reproduction, or whether it is self-compatible and capable of autonomous self-pollination.

2) To assess the relative contribution of bird and bee visitors to the pollination of *A. reitzii*.

3) To infer the relative importance of generalist and specialist nectarivores in the pollination of *A. reitzii*.

4) To investigate the effect of phytophagous and/or parasitoid insects on the reproductive output of *A. reitzii*. The extent of parasitism/predation of *A. reitzii* individuals and the population was also determined.

DISSERTATION OUTLINE

This dissertation comprises five chapters. This first chapter consists of a general introduction, including a literature review, the rationale for the study, an account of the study species, *Aloe reitzii* var. *reitzii*, and the study's aims and objectives. Chapter Two addresses the first objective, to investigate the breeding system of *A. reitzii* using controlled hand-pollination experiments to infer whether *A. reitzii* is dependent on outcrossing, and bagged and open treatments to infer whether *A. reitzii* can autonomously self-pollinate. Chapter Three addresses the second and third objectives, to infer the relative effectiveness of bird and bee visitors on the pollination of *A. reitzii,* and to deduce the relative importance of generalist and specialist nectarivores in the pollination of *A. reitzii*. Visitation rates of birds and insects, pollen loads, and pollinator exclusion experiments were used to assess the relative contributions of birds and insects to the reproductive output (fruit set and seed set) of *A. reitzii*. The relative importance of generalist and specialist nectarivores to *A. reitzii* was inferred by investigating nectar properties, along with visitation rates of bird visitors, main pollen deposition sites, and the relationship between the aloe's perianth tube morphology (length and curvature) and bill morphology (length and curvature) of specialist and generalist nectarivores. Chapter Four addresses the effect of phytophagous and/or parasitoid insects on the reproductive output of *A. reitzii*, by identifying phytophagous and parasitoid wasps and flies emerging from fruits and seeds, and assessing their impact on the reproductive output of *A. reitzii.* The fifth and final chapter is a brief overview of the reproductive ecology of *A. reitzii* with consideration of its reproductive biology and pollination biology, conclusions and future prospects. This dissertation has been prepared and written in the format of scientific papers, and although effort was made to minimize repetition, repetition of some information was unavoidable.

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Appendix A

Table of South African aloes summarizing their flowering period, breeding system and pollination biology; with particular focus on their floral traits (flower colour and floral tube length), nectar properties (nectar volume and concentration), pollination syndrome, and primary pollinator.

***Aloe* species**	**Flowering period**	**Breeding system**	**Flower morphology: colour; shape; (floral tube length)[1]**	**Nectar traits**	**Pollination syndrome**	**Primary pollinator**	**References**
Aloe africana Mill.	Jul – Sep	Self-incompatible	Orange to yellow Long, tubular, fused	Vol: ~ 125 μL; Conc: ~12.5%	Bird	Specialist and generalist nectarivores	Botes *et al*., 2008; Botes *et al*., 2009a
Aloe arborescens Mill.	May – Jul	Self-incompatible	Orange Long, narrow, and tubular	Vol: 39.6 μL Conc: 12.1%	Bird	Specialist nectarivores; Bees (Honeybees *Apis mellifera*)	Hargreaves *et al*., 2012
Aloe boylei Baker.	Nov – Feb	Self-incompatible	Orange Long, narrow and tubular	Vol: 19.4 μL Conc: 22.1%	Bird	Birds (specialist or generalist)[2]; Bees (Solitary bee *Amegilla natalensis*)*	Hargreaves *et al*., 2012
Aloe dominella Reynolds	Jun – Oct	Self-incompatible	Yellow Short and tubular	Vol: 4.5 μL Conc: 20.5%	Bird	Bees (Honeybees *Apis mellifera*)§	Hargreaves *et al*., 2012
Aloe ferox Mill.	May – Sep	Self-incompatible	Orange Medium-length, tubular	Vol: 81.3 μL Conc: 8.6%	Bird	Specialist and generalist nectarivores; Bees (Honeybees *Apis mellifera* and Cape honeybees *Apis mellifera capensis*)	Hoffman, 1988; Stokes and Yeaton, 1995; Botes *et a*l., 2008; Botes *et al*., 2009a; Hargreaves *et al*., 2012; Kuiper *et al*., 2015; Diller *et al*., 2019

***Aloe* species**	**Flowering period**	**Breeding system**	**Flower morphology: (colour; shape; floral tube length)[1]**	**Nectar traits (volume; concentration)**	**Pollination syndrome**	**Primary pollinator**	**References**
Aloe greatheadii var. *davyana* (Schönland) Glen & D.S. Hardy	Jun – Aug	Self-incompatible	Pinkish-red to red Long, tubular (28.1 ± 1.65 mm)	Vol: 14.7 ± 7.1 μL Conc: 18.6 ± 2.7%	Bird	Bees (Honeybees *Apis mellifera*)	Hargreaves *et al*., 2012
Aloe inconspicua Plowes	November	Self-incompatible	Whitish-green Short, tubular	Very little nectar produced	Insect	Solitary bees (*Amegilla fallax*)+	Hargreaves *et al.*, 2008; Hargreaves *et al.*, 2012
Aloe kraussii Baker	Nov – Feb	Self-incompatible	Yellow Short, tubular	Vol: 2.2 μL Conc: 19.1%	Bird	Specialist nectarivores	Hargreaves *et al.*, 2012; Hargreaves *et al.*, 2019
Aloe linearifolia A.Berger	Late Jan – Mar	Self-incompatible	Bright greenish yellow Short, tubular (8 – 12 mm)	Conc: 26.6 ± 1.4%	Insect	Solitary and sub-social bees (*Amegilla* sp. and Honeybees *Apis mellifera*)	Botes *et al*., 2009b
Aloe lineata (Aiton) Haw. var. *muirii* (Marloth)	May – Nov	Self-incompatible	Pink to deep red Long, tubular	Vol: ~25 μL Conc: ~17%	Bird	Specialist nectarivores +; Bees (Cape Honeybee *Apis mellifera capensis*) †	Botes *et al*., 2008; Botes *et al*., 2009a
Aloe maculata Medic.	May – Aug	Self-incompatible ◊	Orange Long, narrow, tubular	Vol: 32.2 μL Conc: 16.6%	Bird	Specialist nectarivores; Honeybees (*Apis mellifera*)	Hargreaves *et al*., 2010; Hargreaves *et al.*, 2012
Aloe marlothii A. Berger	May/June - Aug/Sep	Self-incompatible	Yellow-orange Medium-length, narrow, tubular	Vol: 46.6 μL Conc: 8.3%	Bird	Generalist nectarivores	Symes and Nicolson, 2008b; Symes *et al*., 2009; Hargreaves *et al*., 2012

***Aloe* species**	**Flowering period**	**Breeding system**	**Perianth morphology: colour; shape (floral tube length)[1]**	**Nectar traits (volume; concentration)**	**Pollination syndrome**	**Primary pollinator**	**References**
Aloe minima Baker.	Late Jan – Mar	Self-incompatible	Light pinkish cream Short, tubular (8 – 12 mm)	Conc: 25.9 ± 1.9%	Insect	Solitary and sub-social bees (*Apis mellifera* and *Amegilla* sp.)	Botes *et al*., 2009b
Aloe peglerae Schonl.	Jul – Aug	Self-incompatible X;◊	Red in bud and pale greenish yellow when mature Long, tubular	Vol: 47 ± 7 µL Conc: 10.2 ± 0.5	Bird	Generalist nectarivores; Rodents (Namaqua rock mouse *Micaelamys namaquensis* and eastern rock sengi *Elephantulus myurus*)	van Wyk and Smith; 2008; Botes *et al*., 2009b; Hargreaves e*t al*., 2012; Arena *et al*., 2013; Payne *et al*., 2016; Payne *et al*., 2019
Kumara (Aloe) plicatilis (L.) G.D.Rowley	Aug – Oct	Self-incompatible X	Scarlet Long, tubular (± 5 cm)	Conc: 28.1 ± 12%	Bird	Bees (Honeybees *Apis mellifera*)	Cousins *et al*., 2013
Aloe pluridens Haw.	May – Nov	Self-incompatible	Orange to pinkish red, yellow Long, tubular	Vol: ~75 µL Conc: ~15%	Bird	Specialist nectarivores+; Bees (Cape Honeybee *Apis mellifera capensis*) †	Botes *et al*., 2008; Botes *et al.*, 2009a
Aloe pruinosa Reynolds	Feb – Mar	Self-incompatible	Dark pinkish to red	Vol: 14.8 µL Conc: 19.7%	Bird	Specialist nectarivores; Bees (*Amegilla atrocincta*)†	Wilson *et al*., 2009
Aloe speciosa Baker	Aug – Sep	Self-incompatible	Red, green with white stripes when mature Short, tubular	Vol: ~180 µL Conc: ~7%	Bird	Generalist nectarivores	Botes *et al*., 2008; Botes *et al.*, 2009a
Aloe tenuior Haw.	Year round Late winter (Aug; peak)	Self-incompatible	Orange Short, tubular	Vol: 1.2 µL Conc: 29.6%	Bird	Bees (Pollen-collecting allodapine bees)	Hargreaves *et al*., 2012

***Aloe* species**	**Flowering period**	**Breeding system**	**Flower morphology (colour; floral tube length)^1**	**Nectar traits (volume; concentration)**	**Pollination syndrome**	**Primary pollinator**	**References**
Aloe thraskii Baker	May – Jul (Peak in Jun)	Self-compatible	Bright yellow Short, tubular		Bird	Generalsit nectarivores; Honeybees (*Apis mellifera*) and solitary bees (*Hylaeus* spp.)	Patrick *et al*., 2018
Aloe vryheidensis Groenew.	Jul – Aug	Self-incompatible	Yellow Short, open, campanulate flowers	Vol: 41.1 μL Conc.: 11.6%	Bird	Generalist nectarivores	Johnson *et al.*, 2006; Hagreaves *et al.*, 2012

1 Floral tube measurements not included in the table are within the ranges: long (30 – 33 mm); medium – length (23 – 26 mm); short (8 – 15 mm; Botes *et al.*, 2009b; Hargreaves *et al.*, 2012).

2 Not specified whether generalist or specialist nectarivores as primary pollinators

X: no experimental evidence

◊: Autonomous self-pollination observed

* Not exclusive pollinators

\+ Exclusive pollinators

† Secondary pollinators

CHAPTER TWO

Reproductive biology of the summer-flowering *Aloe*, *Aloe reitzii* var. *reitzii* (Asphodelaceae)

ABSTRACT

Angiosperms have evolved a diverse array of mating strategies due to distinctive features of their biology, such as immobility, which has resulted in the requirement of pollen vectors for efficient pollination, enabling the evolution of diverse floral adaptations. Floral adaptations, namely floral design and floral display have enabled plants to govern their mating opportunities through pollinator attraction and pollen dispersal. However, many plants have evolved large floral displays that attract pollinators which, together with the simultaneous display of multiple open flowers in an hermaphroditic plant, increases the potential for geitonogamous self-pollination. Many angiosperms have evolved self-incompatibility which prevents self-fertilization, requiring outcrossing by pollen vectors for successful seed set. This study investigated the dependence of *Aloe reitzii* var. *reitzii* on pollinators for reproduction; a species frequently visited by both birds and bees. In addition, controlled hand-pollination experiments were used to determine whether *Aloe reitzii* var. *reitzii* is self-compatible. Contrary to most aloes, *Aloe reitzii* var. *reitzii* is shown here to be self-compatible but is reliant on pollinators to transfer pollen between flowers. Bees are shown to facilitate self-pollination in *Aloe reitzii* var. *reitzii,* suggesting a mixed mating system. However, since *Aloe reitzii* var. *reitzii* is not capable of autonomous self-pollination, mixed mating does not provide any reproductive assurance, with pollinator-mediated geitonogamy causing reduced fecundity in *Aloe reitzii* var. *reitzii*. Self-compatibility in *Aloe reitzii* var. *reitzii* provides no reproductive advantage and may have evolved in its lineage due to its restricted distribution and increasing population fragmentation due to anthropogenic change.

KEYWORDS: Geitonogamy; Mixed mating; Ornithophilous; Self-compatibility; Self-pollination

INTRODUCTION

Angiosperms have evolved a diverse array of mating strategies due to distinctive features of their biology, such as immobility, modularity (clonal growth or large plant size) and hermaphroditism; together these add to the complexity of plant breeding systems (Barrett, 2003). The immobility of plants has resulted in the requirement of pollen vectors for efficient pollination, promoting the evolution of diverse floral adaptations (Harder and Barrett, 1996; Barrett, 2003). For example, plants have developed various floral and inflorescence displays linked to particular pollinators, mainly through size variation and morphological complexity, and the extensive diversification of these features in plants can be attributed to the need for successful mating (Harder and Barrett, 1996; Barrett, 2003; Harder and Johnson, 2005).

Floral evolution generally involves two classes of adaptations that promote mating success, namely floral design and floral display, which enable plants to influence their mating opportunities through pollinator attraction and pollen dispersal (Harder and Barrett, 1996; Armbuster, 2014). Since effective cross-pollen dispersal largely depends on the types of pollinators visiting flowers and manipulation of their foraging behaviour by plant reproductive traits (Barrett and Harder, 1996; Barrett, 2003; Mitchell *et al.*, 2009), morphological traits that characterize floral design (structure, colour, scent and nectar production of individual flowers; Barrett and Harder, 1996) and floral display (number of flowers open simultaneously and their arrangement; Barrett and Harder, 1996) modify the foraging behaviour of pollen vectors to enhance fecundity (Harder and Barrett, 1996; Mitchell *et al.*, 2009). Hence, floral traits exhibited by animal-pollinated angiosperms, ensure efficient cross-pollen transfer by pollen vectors (Harder and Barrett. 1996). However, these characteristics may also allow pollination between flowers on the same individual (geitonogamous self-pollination), particularly by the simultaneous display of many flowers, which is important for pollinator attraction (Harder and Barrett, 1995; Barrett, 2003).

To attract pollinators, many plants have evolved large floral displays, such as those commonly seen in bird-pollinated plants (e.g., *Kniphofia* Moench, Brown *et al.*, 2010, and *Aloe* L., Stokes and Yeaton, 1995). Large floral displays in hermaphroditic plants, together with the simultaneous presentation of male and female gametes in open flowers, increase the potential for geitonogamous self-pollination (i.e., inter-flower fertilization; Harder and Barrett, 1995), which results in reduced genetic variability, subsequently reducing plant fitness through inbreeding depression and pollen discounting (Harder and Barrett, 1996; Richards, 1997;

Barrett, 2002, 2003). To limit selfing due to the proximity of male and female sex organs, hermaphroditic plants have evolved various anti-selfing mechanisms, such as self-incompatibility (Seavey and Bawa, 1986), and the spatial (herkogamy; Webb and Lloyd, 1986) and/or temporal (dichogamy; Lloyd and Webb, 1986) separation of male and female gametes (Barrett, 2002, 2003).

To prevent self-pollination, many angiosperms have evolved self-incompatibility mechanisms (Barrett, 1988), which reduce the costs of geitonogamy through ovule discounting (loss of seed production when self-pollen disables ovules through early inbreeding or late-acting self-incompatibility; Duffy *et al.*, 2013). Outcrossing by pollinators in self-incompatible plants is shown to ensure successful reproductive output by reducing ovule discounting (Duffy *et al.*, 2013), thereby mitigating the costs of geitonogamy associated with large floral displays and the simultaneous presentation of male and female gametes. For example, *Kniphofia linearifolia* Baker (Asphodelaceae), similar to many species in its genus (e.g., *K. caulescens* Baker, Brown *et al.*, 2009 and *K. laxiflora* Kunth, Brown *et al.*, 2010), is self-incompatible, thus requiring outcrossing by pollen vectors for seed set (Duffy *et al.*, 2013). However, pollen discounting (reduced amounts of cross-pollen reaching conspecifics as a result of self-pollination; Holsinger *et al.*, 1984) can still occur in self-incompatible plants, reducing male fitness as no seeds are sired (Harder and Barrett, 1995), while ovule discounting by self-pollen reduces female fitness (Duffy *et al.*, 2013).

Many aloes have floral traits consistent with a bird (ornithophilous) pollination syndrome, with large floral displays associated with pollinator attraction (Stokes and Yeaton, 1995). Aloes have evolved various anti-selfing mechanisms that may serve to reduce the probability of geitonogamous self-pollination associated with their large floral displays, and the proximity of male and female gametes. These include self-incompatibility, viz., ovarian (Hargreaves, 2007; Hargreaves *et al.*, 2012), protandry (Botes *et al.*, 2008; Hargreaves *et al.*, 2012), herkogamy (Hargreaves *et al.*, 2012) and the acropetallous maturation of flowers on the raceme (Botes *et al.*, 2008).

Self-incompatibility is widespread in the genus *Aloe* (Cousins *et al.*, 2013), with most *Aloe* species studied to date found to be self-incompatible, requiring outcrossing by pollen vectors for successful seed set (e.g. *A. ferox* Mill., Hoffman, 1988; *A. inconspicua* Plowes, Hargreaves *et al.*, 2008; *A. marlothii* A.Berger, Symes and Nicolson, 2008; *A. linearifolia*

A.Berger, Botes *et al.*, 2009; *A. pruinosa* Reynolds, Wilson *et al.*, 2009; Appendix A, Chapter 1). Although a few aloe species appear capable of autonomous self-pollination, such as *A. maculata* Medic. and *A. kraussii* Baker (Hargreaves *et al.*, 2012), *A. peglerae* (Arena *et al.*, 2013) and *Kumara plicatilis* (L.) G.D. Rowley (Cousins *et al.*, 2013), all species had low seed set when self-pollinated (Hargreaves *et al.*, 2012; Arena *et al.*, 2013). Self-compatibility has been observed in few *Aloe* species (e.g., *A. divaricata* Berger, Ratsirarson, 1995; *A. mayottensis* A.Berger, Pailler *et al.*, 2002). However, *Aloe thraskii* Baker is the only South African aloe to date that is known to be self-compatible, but it still depends on pollen vectors to transfer pollen between flowers (Patrick *et al.*, 2018). Self-incompatibility in aloes is a result of mechanisms acting in the ovary (late-acting self-incompatibility; Seavey and Bawa, 1986; Hargreaves *et al.*, 2012), as observed in *A. maculata* (Hargreaves, 2007). Therefore, self-incompatibility in aloes enables them to maintain a high seed set through reduced ovule discounting by relying on cross-pollen transfer by pollen vectors for successful seed set (Duffy *et al.*, 2014). Animal pollinators therefore play a crucial role in the fecundity of aloes, hence investigating breeding systems in *Aloe* is important in determining the diversity and success of plant-pollinator interactions (Arena *et al.*, 2013). This may contribute to the management and conservation of wild *Aloe* populations and their respective environments (Cousins and Witkowski, 2012).

Similar to many *Aloe* species, *Aloe reitzii* Reynolds var. *reitzii* (hereafter referred to *A. reitzii*) has floral traits consistent with an ornithophilous pollination syndrome. Since most *Aloe* species are self-incompatible, they require pollen vectors for successful seed set (Hoffman, 1988; Hargreaves *et al.*, 2008; Symes and Nicolson, 2008; Hargreaves *et al.*, 2012; Appendix A, Chapter 1). Although birds and bees are abundant at *A. reitzii* flowers (Symes, 2017; Payne, 2019), the reliance of *A. reitzii* on cross-pollen transfer by animal vectors for reproduction has not yet been investigated. Therefore, this study aimed to determine whether *A. reitzii* is dependent on pollen vectors for successful seed set by investigating its breeding system. Controlled hand-pollination experiments were conducted to infer whether *A. reitzii*, similar to many species in its genus, is self-incompatible, and bagged and open treatments were used to test if it is capable of autonomous self-pollination.

MATERIALS AND METHOD

Study species and study site

Aloe reitzii is a stemless aloe, reaching a height of up to 1 m (Symes, 2017), with a mean rosette height (leaf height) of 66.59 ± 1.12 cm, and a mean rosette diameter of 60.55 ± 1.15 cm (Payne, 2019a; Figure 2.1A). It is characterized by dark red flowers that turn yellow as they mature (Van Wyk and Smith, 2008). They have distinctly curved, long, (up to 50 mm long; Table 2, Chapter 3), tubular, downward pointing flowers, which are used to distinguish this Near-Threatened (Mtshali *et al.*, 2018a) species from similar species (e.g., *A. aculeata* Pole-Evans, *A. gerstneri* Reynolds and *A. petricola* Pole-Evans; Van Wyk and Smith, 2008). *Aloe reitzii* plants have on average three racemes, with up to ten racemes observed on a single plant (Symes, 2017).

Contrary to most ornithophilous aloes, *Aloe reitzii* flowers in summer — between February and March (Van Wyk and Smith, 2008). It is endemic to the rocky slopes and grasslands in regions around Roossenekal and Belfast to the Barberton Mountains in Mpumalanga (Mtshali *et al*., 2018a), but localised distributions in Limpopo and Gauteng have also been noted (Van Wyk and Smith, 2008). The spring-flowering variety, *Aloe reitzii* Reynolds var. *vernalis* D.S.Hardy, is Vulnerable and is known from a single isolated population in northern KwaZulu-Natal (Mtshali *et al.*, 2018b).

The study was conducted on Klipbankspruit Farm (25°16'21.9" S, 30°3' 14" E; ~ 1840 m.a.s.l.) approximately 30 km north-east of Dullstroom, Mpumalanga, where *Aloe reitzii* grows in abundance (Figure 2.1B). The privately-owned farm is used for commercial beef farming and grass bailing (Symes, 2017; Payne, 2019a). Livestock, such as cattle, donkey and sheep, graze on sites where the *Aloe reitzii* is found (Payne, 2019a).

Breeding system

The study took place during the peak of one flowering season in February 2020. To test whether *A. reitzii* is dependent on floral visitors for pollination and subsequent seed set or whether it is capable of autonomous self-pollination, open and bagged treatments were used. Controlled hand-pollination experiments were conducted to test whether *A. reitzii* relies on outcrossing for reproduction, and whether it is self-compatible. Racemes on 15 randomly selected aloes, each with ≥ four racemes, were assigned to one of the following treatments: 1) Bagged, flowers unmanipulated, testing for autonomous self-pollination, 2) selfed, in which the stigma of selected flowers received pollen from another flower on the same raceme (geitonogamy), 3) crossed, in which the stigma of selected flowers received pollen from a flower on another plant (allogamy; Botes *et al.*, 2009), and 4) open (control), receiving pollen from all floral visitors. For controlled hand-pollination experiments, five flowers were randomly selected per raceme across 15 aloes, resulting in a total of 75 flowers per treatment. To compensate for flowers that fell off the raceme following hand-pollination (likely due to damage), new flowers were continuously pollinated to ensure each raceme had five hand-pollinated flowers at the end of the flowering season.

For all treatments, racemes were bagged (to exclude pollinators) before anthesis with fine organza bags, i.e. while still in bud. Selected racemes were marked by tying blue (bagged), green (crossed) and yellow (selfed) ribbons around the raceme stalk to indicate the treatment (Figure 2.1C). Selected flowers for hand-manipulated self- and cross-pollinations were marked using white correction fluid on the corollas and pedicels, and later marked with blue ink when pollinated (Figure 2.1D). All flowers were counted for the open (control) and bagged treatments. To avoid contamination of stigmas with self-pollen, flowers used in the self- and cross-pollination treatments were emasculated before anthesis (Wilson *et al.*, 2009; Duffy *et al.*, 2013). Hand-pollination crosses were conducted by brushing dehisced anthers across receptive stigmas as soon as they had elongated and their surface area expanded (Patrick *et al.*, 2018).

Near the end of the flowering season (late March), fruits from all treatments were collected. Fruit and seed set were determined, the latter after the fruit had dehisced at ambient temperatures. To determine fruit set and seed set, only mature fruit and seeds were used. Seed set from plants where no fruit was produced was excluded from data analyses. Differences in fruit and seed set were then compared between self- and cross-pollinated treatments, the bagged and open treatments. Overall fruit set for various treatments was determined using the following calculations, adapted from Payne (2019a):

Bagged and open treatments:

$$\text{Fruit set (\%)} = \frac{\text{No. of total fruit produced/raceme}}{\text{No. of total flowers/raceme}} \text{ x } 100 \qquad *$$

* Number of flowers were represented by the number of pedicels present after flowers had fallen off

Self- and cross-pollination treatments:

$$\text{Fruit set (\%)} = \frac{\text{No. of fruit produced (from pollinated flowers)}}{\text{No. of hand-pollinated flowers/raceme}} \text{ x } 100$$

Figure 2.1. A) Flowering (mature) *Aloe reitzii* var. *reitzii* plant; B) Population of flowering *Aloe reitzii* var. *reitzii* plants at the study site, Klipbankspruit Farm; C) Bagged, cross- and self-pollinated treatments of *Aloe reitzii* var. *reitzii* represented by blue, green and yellow ribbons, respectively, while open treatments (no bag present) allowed all floral visitors; D) Marked flowers in the self- and cross-pollination treatments used in the hand-pollination experiments. Scale bar = 10 cm. Photographs by Jessica Minnaar.

Germination trials and seed viability

To test for seed viability of open and bagged treatments, five fruits were randomly selected from each treatment raceme and a maximum of 10 seeds were randomly selected per fruit, resulting in a maximum of 50 pooled seeds per raceme. Only mature seeds (dark grey in colour) were selected. Similarly, for hand-pollination experiments, seed viability tests were conducted by randomly selecting 10 seeds from each pollinated fruit, resulting in a maximum of 50 pooled seeds per raceme (where possible). For each raceme, 10 seeds were randomly selected from these 50 pooled seeds, resulting in a total of 150 seeds per treatment (N = 15 aloes/treatment). Seeds were placed into sterilized Petri dishes on top of two filter papers. To prevent fungal growth during seed germination, the filter papers were washed in 2% bleach, followed by a double rinse in distilled water (Arena *et al.*, 2013). The seeds were covered with one filter paper and saturated with distilled water. The Petri dishes were kept in an environmental control chamber, with daytime temperatures of 25 °C and night-time of 15 °C (12 h day-night cycle; Arena *et al.*, 2013; standard germination conditions, S_{GC}). Germinating seeds, identified by a 2 mm radicle protruding from the seeds, were counted every day for three weeks, and thereafter, three times a week for an additional three weeks (Arena *et al.*, 2013; Payne *et al.*, 2016).

Seeds that had not germinated were removed from the control chamber and empty seed coats were removed and recorded as "dead". To determine seed viability of the ungerminated seeds, any intact seeds were sliced in half to expose the embryo and saturated with a 1.0 % 2,3,5-triphenyltetrazolium chloride (tetrazolium) solution overnight (Arena *et al.*, 2013; Payne *et al.*, 2016). Seeds with embryos that had turned pink the following day were considered to be "viable", while seeds with embryos that remained white were recorded as "non-viable" (ISTA, 2003). Total seed viability (%) was calculated as the sum of the percentage of seeds that germinated and those that were viable after tetrazolium seed testing (Payne *et al.*, 2016).

Since many seeds did not successfully germinate under standard conditions, seeds were also germinated in ambient conditions (room conditions, R_{GC}). For each treatment, 50 seeds were randomly selected from pooled seeds/raceme and germinated (as outlined above). The Petri dishes were kept in a relatively well-lit environment, at room temperature (~17 – 25 °C; ~13.5 h , 10.5 h day-night cycle).

Data analysis

A non-parametric Kruskal-Wallis test was used to test for differences in i) fruit set and seed set among treatments, and ii) percentage germination, seed viability (tetrazolium tests) and total seed viability among treatments (Shapiro-Wilks Normality Test, $P < 0.05$). *Post-hoc* analysis was conducted on significant results using the Kruskal-Wallis Multiple Comparison *Post-hoc* Test (R package ‘pgirmess’; Giraudoux *et al.*, 2018). Statistical analyses were done in R (v. 4.0.0; R Core Team, 2020).

RESULTS

Breeding system

To test if *A. reitzii* is dependent on pollinators for reproduction and subsequent seed set, and can autonomously self-pollinate, open and bagged treatments were used, and controlled hand-pollination experiments were conducted to determine whether *A. reitzii* is self-compatible.

More fruit were produced in open (23.78 ± 17.59%; $N = 15$) than in bagged (4.51 ± 5.66 %; $N = 15$) treatments. Hand-crossed treatments (20.64 ± 3.51%; $N = 15$) produced more fruit compared to hand-selfed (8.34 ± 12.27%; $N = 15$) treatments. Fruit set did not differ significantly among treatments ($H = 7.44$, df = 3, $P > 0.05$; Figure 2.2A). Coefficients of variation (CV) were 74%, 125%, 38% and 147% for open, bagged, hand-crossed and hand-selfed treatments, respectively. The high CV’s explain the lack of statistical significant difference among treatments resulting from the high degree of dispersion of data around the mean. This could be explained by an aloe that did not produce any fruit for open, bagged and crossed treatments, and only produced three fruits for selfed treatments. After removing this aloe, fruit set differed significantly among treatments ($H = 9.45$, df = 3, $P < 0.05$), with fruit set differing between open and hand-selfed treatments ($P < 0.05$).

Although low seed set was observed in all treatments, seed set was highest in crossed (37.61 ± 16.62 seeds per fruit) and open treatments (23.62 ± 13.77 seeds per fruit), with lowest seed set recorded in selfed (15.64 ± 14.23 seeds per fruit) and bagged treatments (7.28 ± 8.52 seeds per fruit). Seed set differed significantly among treatments ($H_2 = 17.45$, df = 3, $P < 0.001$), with only bagged treatments producing significantly fewer seeds compared to crossed ($P < 0.05$) and open ($P < 0.05$; Figure 2.2B) treatments. Removing the aloe that produced no fruit (and no seeds) did not change seed set results. These results suggest that *A. reitzii* is self-compatible.

Germination trials and seed viability

Seed germination trials were conducted to infer seed viability of open and bagged treatments, and the controlled hand-pollination treatments. In standard germination conditions (S_{GC}), seeds only began germinating after 20 days (Figure 2.3). Percentage germination was relatively low across all treatments, with the exception of hand-crossed treatments, but it was highly variable, ranging from 0 –70% in crossed treatments, 0 – 30 % in bagged treatments, and 0 – 20% in open treatments. No seeds germinated in the selfed treatments. Percentage germination was highest in open (13.0 ± 2.13% seeds; $N = 100$) and crossed (12.86 ± 9.93% seeds; $N = 62$) treatments and was lowest in the bagged treatment (6.03 ± 3.49% seeds; $N = 80$; Figure 2.4). Percentage germination differed significantly among treatments ($H = 12.5$, df = 3, $P < 0.01$), with significantly higher percentage germination in open treatments compared to selfed treatments ($P < 0.05$). Seed viability of the ungerminated seeds (tetrazolium testing) did not differ significantly among treatments ($H = 2.31$, df = 3, $P > 0.05$). Total seed viability (germinated and tetrazolium testing combined) did not differ significantly among treatments ($H = 7.62$, df = 3, $P = 0.05$). CV's for total seed viability for open, bagged, crossed and selfed treatments were 46%; 167%; 109%; 110%, respectively. The high CV's indicate a high dispersion around the mean, explaining the lack of statistical difference among treatments.

A higher percentage of seeds germinated per treatment when seeds were placed in a relatively sunlit environment (Room germination conditions (R_{GC}); Figure 2.4). When seeds were germinated in a relatively sunlit environment at ambient temperature (~17 – 25 °C), seeds from all treatments germinated within four days. Percentage germination was highest in crossed (78%; $N = 50$) and selfed (65%; $N = 38$), and lowest in open (50%; $N = 50$) and bagged (32%; $N = 50$) treatments (Figure 2.4). Viability tests for seeds that had not germinated showed that only a small percentage of these seeds were viable for the selfed (14.29%) and bagged (4.17%) treatments, with none in the crossed and open treatments (Figure 2.4).

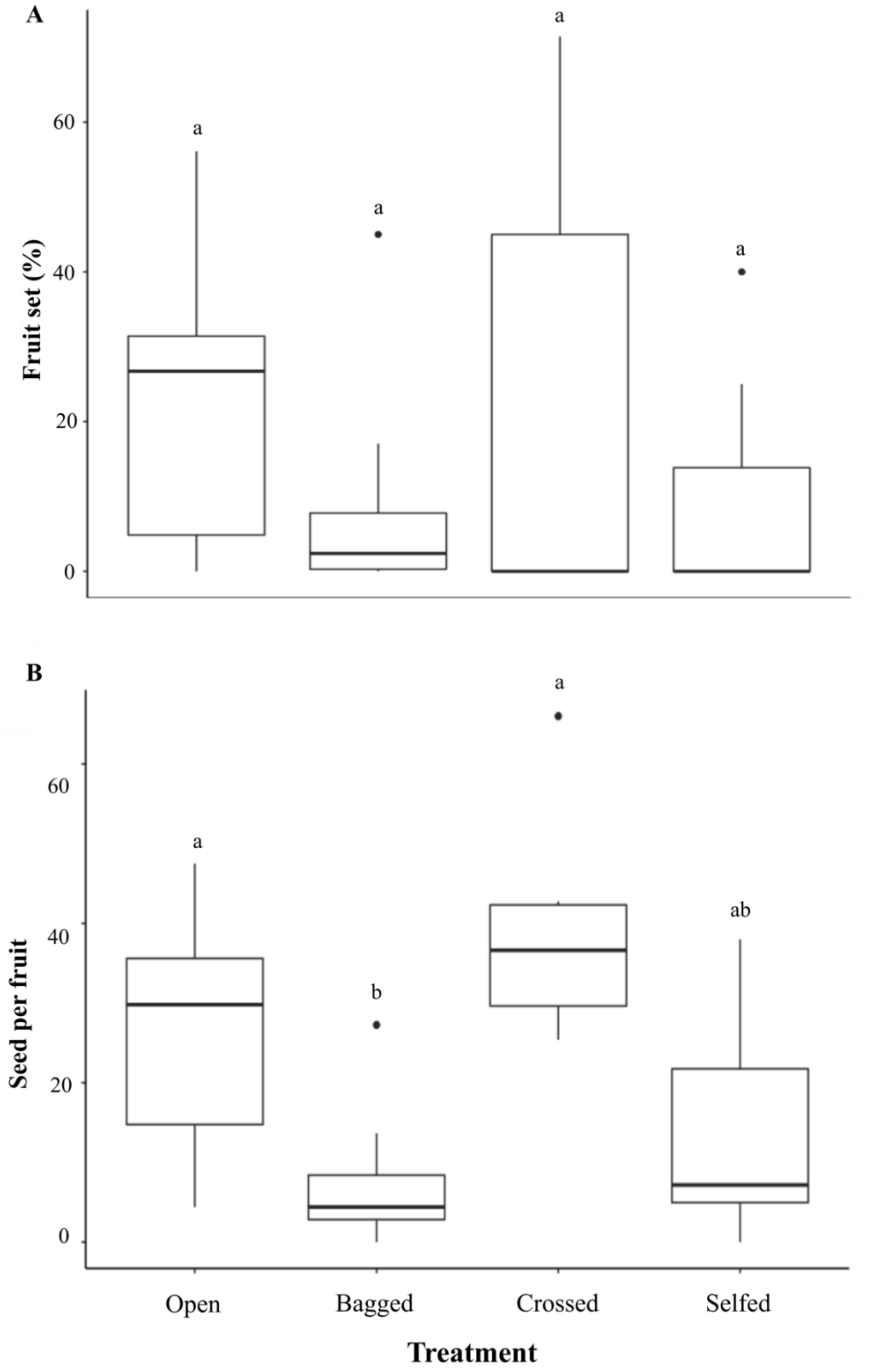

Figure 2.2. Reproductive success of *Aloe reitzii* var. *reitzii* for open and bagged treatments, and hand-pollination experiments during February to March 2020 flowering season (open, bagged, crossed and selfed treatments), as measured by, A) fruit set (proportion of flowers that set fruit) and B) seed set (number of seeds/fruit). These results are inclusive of the aloe that produced zero fruit. Boxes represent median and inter-quartile range, bars represent minimum and maximum, and solid circles represent outliers. Treatments with different letters are significantly different (Kruskal-Wallis Multiple Comparison, $P < 0.05$).

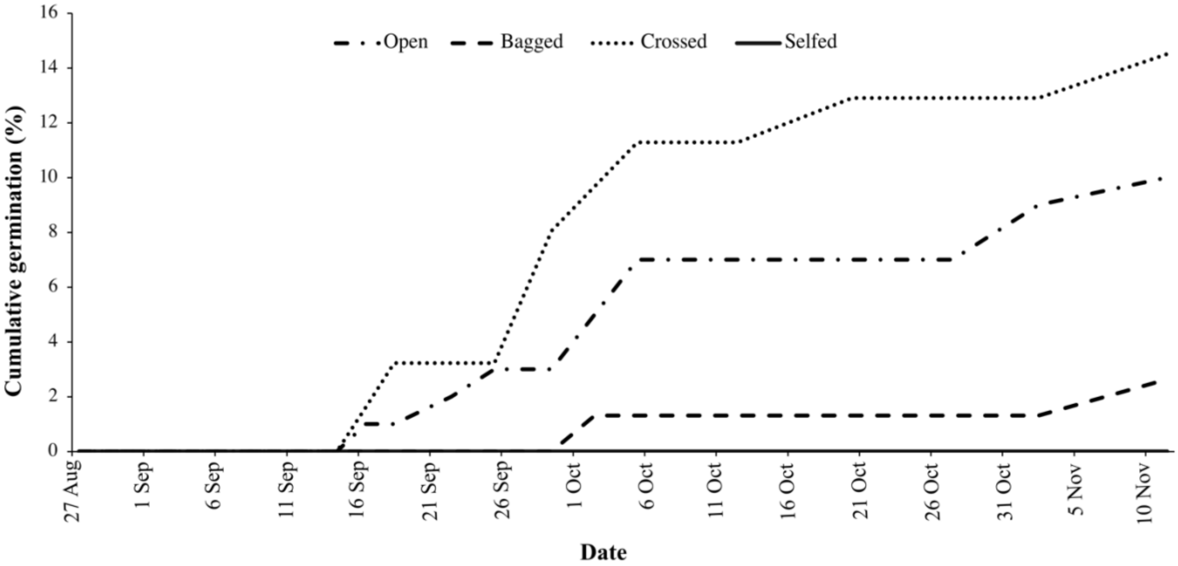

Figure 2.3. Cumulative percentage germination over the 11-week germination trial in standard germination conditions [daytime temperatures of 25 °C and night-time temperatures of 15 °C (12 h day-night cycle)] between the four breeding system experiments (open, bagged, crossed and selfed treatments) on *Aloe reitzii* var. *reitzii*. No seeds germinated for the hand-selfed treatment.

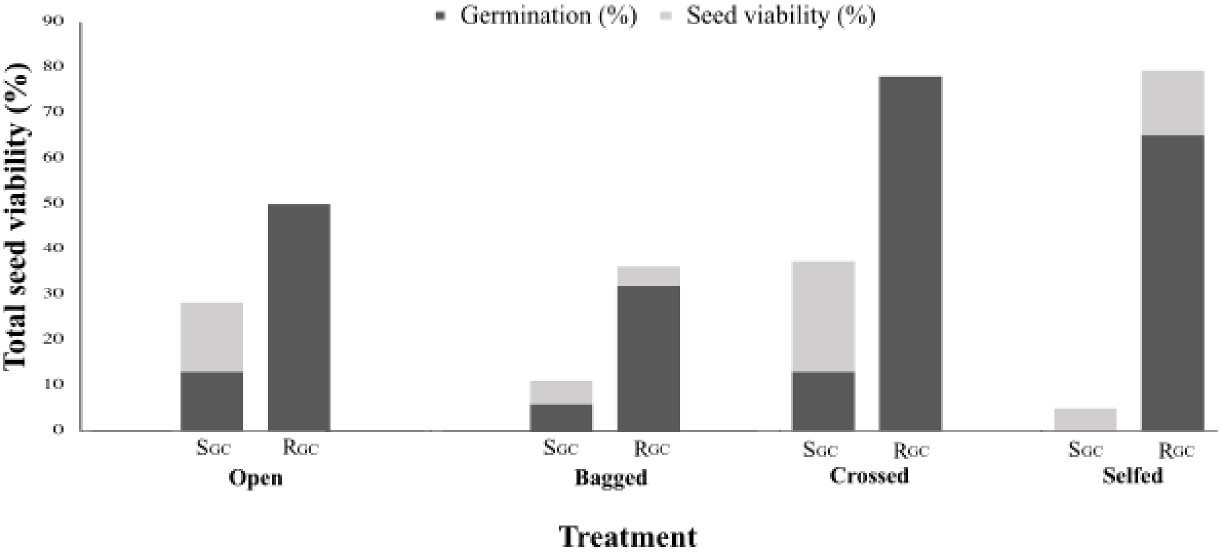

Figure 2.4. Total seed viability (%), as shown by the sum of percent seed germination and viability of ungerminated seeds, for each treatment under standard germination conditions [$S_{GC;}$ daytime temperature of 25 °C and night-time temperatures of 12 °C (12 h day-night cycle)] and ambient germination conditions ($R_{GC;}$ ~17 – 25 °C; ~13.5h, 10.5 h day-night cycle)]. Only mature seeds were used for germination trials and seed viability tests.

DISCUSSION

Our study investigating the reliance of *A. reitzii* on pollinators for reproduction shows that it is not capable of autonomous self-pollination; thus *A. reitzii* is dependent on pollinators for pollen transfer between flowers. This is evidenced by the lower fruit set and seed set, as well as low seed viability in the bagged treatments. Baker and Cruden (1991) showed that insects, namely thrips and aphids, can mediate self-pollination. Thrips were common visitors to *A. reitzii* flowers (Table 2, Chapter 3), and along with a variety of small beetles, were often observed crawling around flowers of bagged racemes (J. Minnaar, *pers. obs*.). Hence, the presence of these insects inside bagged treatments may have resulted in geitonogamous selfing amongst the protrandrous flowers, rather than autonomous selfing. This would account for the limited fruit set and seed set in bagged racemes. Additionally, rainfall and strong winds resulted in pollen being brushed onto the sides of bags (J. Minnaar, *pers. obs.*) which could have pollinated flowers with receptive stigmas as they acropetally matured. Therefore, it is likely that fruit set and seed set in bagged treatments are a result of geitonogamous selfing, rather than autonomous selfing.

In addition, from the results presented here, it appears that *A. reitzii* is self-compatible; one of the few studies to observe self-compatibility in *Aloe.* Although self-incompatibility is widespread in the genus *Aloe* (Cousins and Witkowski, 2012), self-compatibility is present in a few *Aloe* species, such as *A. thraskii* (South Africa; Patrick *et al.*, 2018) and two island endemics, *A. divaricata* (Madagascar; Ratsirarson, 1995) and *A. mayottensis* (Island of Mayotte, Indian Ocean; Pailler *et al.*, 2002). Similar to *A. reitzii*, these self-compatible aloes require pollinators to transfer pollen between flowers within a raceme and between racemes on a plant (Ratsirarson, 1995; Pailler *et al.*, 2002; Patrick *et al.*, 2018).

Although self-incompatibility is widespread in the family Asphodelaceae, self-compatibility does occur in genera related to *Aloe*, such as *Bulbine* Wolf (e.g., *Bulbine vagans* E.M. Watson; Vaughton and Ramsey, 2010) and *Gasteria* Duval (Smith *et al*., 1992). Self-compatibility is also observed in other “self-incompatible” families such as Proteaceae (Steenhuisen and Johnson, 2012), with self-compatibility common in many grassland *Protea* L. species in the eastern Region of South Africa (Hargreaves *et al.*, 2004; Steenhuisen and Johnson, 2012), as well as in the Cape (Schmid *et al*., 2015). Therefore, self-compatibility in *Aloe* may be more widespread than previously reported, and the breeding system of more *Aloe* species needs to be further investigated.

Since *A. reitzii* is self-compatible, geitonogamy is unavoidable (Lloyd, 1992). Self-compatibility in *A. reitzii*, combined with the large floral displays and the simultaneous opening of many flowers on the same raceme increases the probability of geitonogamous selfing (Harder and Barrett, 1995), making *A. reitzii* susceptible to inbreeding depression due to decreased genetic variability (de Jong *et al*., 1993; Harder and Barrett, 1996), and reduced outcross siring success (Harder and Barrett, 1995). Contrary to self-compatible *A. thraskii* (Patrick *et al.*, 2018), *A. reitzii* experienced reduced fecundity from self-pollen, suggesting possible early inbreeding depression (Harder and Barrett, 1995). Although *A. reitzii* appears to lack self-incompatibility mechanisms to reduce the probability of geitonogamy, *A. reitzii* has floral traits that reduce geitonogamy, such as protandry, herkogamy (stamens elongate further than style; J. Minnaar, *pers. obs*) and flowers that mature acropetally on the raceme (Botes *et al.*, 2008; Hargreaves *et al.*, 2012). These floral traits, mainly protandry and herkogamy, reduce levels of self-pollination and enhance plant fitness by ensuring efficient pollen dispersal (Barrett, 1998). Therefore, these floral traits reduce the probability of geitonogamous selfing, but it is possible that populations of *A. reitzii* may still experience increased levels of inbreeding depression due to pollinator-mediated selfing. However, the level of possible inbreeding depression resulting from self-pollen deposition in *A. reitzii* needs to be quantified by comparing genetic markers in seeds produced by selfed progeny and mature plants.

Pollinators can mediate self-pollination by foraging within flowers (facilitated selfing) or between flowers on the same plant (geitonogamy; Lloyd, 1992, Lloyd and Schoen, 1992). Bees are shown to play a small role in the pollination of *A. reitzii* (Chapter 3), and likely mediate geitonogamous self-pollination, as they forage between flowers on the same raceme and/or plant (J. Minnaar, *pers. obs*). Additionally, birds were often observed feeding between racemes on the same plant (J. Minnaar, *pers. obs*.). Hence, self-pollen, mostly by bees, combined with cross-pollen by birds (Chapter 3), suggests mixed mating (combination of cross- and self-pollen in the same individual; Goodwillie *et al*., 2005) in *A. reitzii*.

Mixed mating has been observed in the self-compatible species *Bulbine vagans* (Vaughton *et al*., 2008; Vaughton and Ramsey, 2010). However, due to delayed autonomous selfing in *B. vagans*, reproductive assurance is provided when cross-pollen is limited (Vaughton *et al*., 2008). Mixed mating is an unavoidable consequence in self-compatible plants with pollinator-mediated geitonogamous selfing (Lloyd, 1992). Since geitonogamy requires pollinators for pollen transfer between flowers, it does not provide reproductive assurance if pollinators are limited (Lloyd, 1992; Lloyd and Schoen, 1992). Hence, a mixed mating system in *A. reitzii* provides no benefits to its reproduction, as pollinator-mediated selfing results in reduced fecundity and possibly also inbreeding depression. However, in plants primarily pollinated by birds (e.g., *Kniphofia linearifolia* Baker, Duffy *et al.*, 2013), the removal of self-pollen by bees where birds are abundant may reduce ovule discounting, (Duffy *et al.*, 2013), thus enhancing fecundity. Since *A. reitzii* is primarily bird-pollinated (Chapter 3), removal of self-pollen by bees may enhance fecundity.

Kniphofia linearifolia, a self-incompatible species also related to the genus *Aloe*, experiences ovule discounting as a result of a mixture of self- and cross-pollen on stigmas (Duffy *et al.*, 2013). However, Duffy *et al.* (2013) suggested that if bees removed self-pollen from the plant, together with an abundance of birds, seed production may be improved by reducing ovule discounting, as more outcrossed pollen will be deposited on stigmas. In *Aloe reitzii*, treatments that allowed insect visitors only (bird exclusion treatments) experienced reduced fecundity compared to treatments that included both birds and bees (open treatments; Chapter 3), suggesting that mediated geitonogamous selfing by bees may cause reduced fecundity. In contrast, treatments that included both birds and bees experienced greater seed production, suggesting self-pollen removal by bees may ensure more outcross pollen deposition, reducing ovule discounting and mitigating the fitness costs associated with geitonogamy.

Self-compatibility is said to have evolved from a significant increase in inbreeding depression resulting from pollinator limitation or population bottleneck (Schemske and Lande, 1985). However, the selection for selfing has to have a fecundity advantage (Schemske and Lande, 1985), thus selfing is commonly interpreted to have evolved to provide reproductive assurance when mates and/or pollinators are limited (Lloyd, 1992). However, both birds and bees are highly abundant visitors to *Aloe reitzii*, and since *A. reitzii* cannot autonomously self-pollinate, no reproductive assurance is provided by self-compatibility (Lloyd, 1992; Lloyd and Schoen, 1992). Therefore, it is possible that self-compatibility in *A. reitzii* may have arisen through changing population dynamics and plant-pollinator interactions. *Aloe thraskii* has a restricted distribution along the east coast of South Africa (Patrick *et al.*, 2018). Self-compatibility in *A. thraskii* is proposed to have evolved due to its history of colonizing sand dunes, since self-compatibility is common in colonizing species with a metapopulation structure (Patrick *et al.*, 2018). Similar to *A. thraskii*, both *A. reitzii* var. *reitzii* and *A. reitzii* var. *vernalis*, have restricted and/or localized distributions, with populations increasingly becoming fragmented due to anthropogenic changes, e.g., commercial timber plantations, mining and quarrying development (Mtshali *et al.*, 2018a,b). Additionally, *A. reitzii* var. *vernalis* is only known from a single locality (Mtshali *et al.*, 2018b). Since selfing is suggested to have evolve in isolated or in small populations which may experience reduced efficiency of cross-pollination (Busch and Delph, 2012), self-compatibility in *A. reitzii* may have arisen through reduced outcrossing due to reduced pollinator abundance. This reduction in number of pollinators can also be caused by habitat fragmentation (Eckert *et al.*, 2009), whereby fragmented populations experience greater levels of inbreeding (Eckert *et al.*, 2009), shifting mating patterns towards increased selfing (Aguiler *et al.*, 2008). Thus, increased selfing levels arising from reduced pollinator abundance may have resulted in a shift in the mating system of *A. reitzii*. However, the breeding system of the very rare variety, *A. reitzii* var. *vernalis*, also needs to be investigated to infer possible life history traits that may have resulted in the evolution of self-compatibility in the species as a whole.

Seed germination and seed viability of *A. reitzii* were very low for both standard (S_{GC}) and room (R_{Gc}) germination conditions compared to other seed germination studies on *Aloe* (e.g., Arena *et al.*, 2013; Cousins *et al.*, 2013; Payne *et al.*, 2016; Kremer-Köhne, 2018), which could be attributed to the timing of fruit collection or to nonoptimal seed germination conditions. Due to exceptional circumstances (i.e., lockdown due to a pandemic), fruits were collected near the end of March instead of early April, two weeks before the fruits dehisced. Therefore, low seed set (and germination) in all treatments could be a result of collecting fruits before the seeds had fully matured. Alternatively, the set environmental conditions may have affected germination rate and viability. Low seed germination and viability were observed when *A. reitzii* seeds were germinated in the same growth conditions as winter-flowering aloes (e.g., *A. peglerae*, Arena *et al.*, 2013, Payne *et al.*, 2016; and *K. plicatilis*, Cousins *et al.*, 2013), which would likely germinate in spring (S. Payne, *pers. comm.*), in warmer temperatures with more frequent rainfall. Since *A. reitzii* fruits dehisce mid- to end-April, seeds are likely to germinate in cooler temperatures with less frequent rainfall since a higher percentage of seeds germinated in cooler (~17 – 25 °C) room temperatures but longer summer daylight hours (~13.5 h, 10.5 h day-night). Additionally, even if germinated in optimal conditions, the presence of fungal growth (also observed in *A. peglerae*; Arena *et al.*, 2013), could also have attributed to the lower seed germination rates seen here. Low seed germination in *A. reitzii* can also be attributed to resource limitation (e.g., soil properties and water availability) during flowering and fruiting periods (Payne, 2019b). Additionally, aloes may invest more in pollen production, rather than in fruit and seeds (Payne, 2019b), resulting in low seed production and germination rates. Despite being hand-pollinated or open for visitation, one aloe did not produce fruit for all treatments. It is not uncommon in *Aloe* for a raceme or whole aloe plant to not produce any fruit, despite receiving conspecific pollen, likely a result of resource limitation (Payne, 2019b).

Conclusion and future recommendations

Aloe reitzii is only the second aloe in South Africa observed to be self-compatible. Similar to the other known self-compatible aloes, *A. reitzii* is probably not capable of autonomous self-pollination and is thus likely to be reliant on pollinators for pollen transfer between flowers. However, to truly assess whether *A. reitzii* is capable of autonomous self-pollination, wire frames should be used in mesh bags to prevent flowers from brushing pollen onto the sides of bags (e.g., Symes and Nicolson, 2008; Arena *et al.*, 2013). Additionally, racemes should be checked for small insects, such as thrips, prior to enclosing them in bags. Self-pollen reduces fecundity of *A. reitzii*, suggesting self-compatibility in *A. reitzii* could result in inbreeding depression. Additionally, pollen discounting and ovule discounting resulting from self-pollen deposition should be quantified through floral emasculation (see Duffy *et al.,* 2013). Pollinator-mediated geitonogamous selfing by bees in *A. reitzii* results in seed discounting and reduced fecundity. However, the amount of self-pollen removal and deposition by bees needs to be quantified. Self-compatibility may have evolved due to the aloe’s restricted distribution, and further investigation of the breeding system of *A. reitzii* var. *vernalis* may provide insight into life history traits of both *A. reitzii* varieties that may have resulted in the evolution of self-compatibility and/or self-incompatibility in these taxa with restricted distributions.

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CHAPTER THREE

Birds or bees for fruit and seeds: key pollinators of the summer flowering *Aloe reitzii* var. *reitzii*

ABSTRACT

Most aloes have floral traits consistent with a bird pollination syndrome, such as their red/orange tubular flowers, exserted filaments and copious nectar production. Despite these ‘ornithophilous’ traits, bees have been shown to be effective pollinators of many summer-flowering *Aloe* species. Previous studies on *Aloe reitzii* var. *reitzii*, a summer-flowering aloe endemic to South Africa, showed that both birds and bees were frequent visitors. This study aimed to investigate the pollinator effectiveness of birds and bees to *Aloe reitzii* var. *reitzii* and to infer the importance of specialist and generalist nectarivores to the aloe’s pollination. Pollen loads between birds and bees did not differ significantly ($P > 0.05$), indicating that both birds and bees play a role in the pollination of *Aloe reitzii* var. *reitzii*. Pollinator exclusion experiments where birds were excluded still set fruits and seeds, suggesting bees successfully transfer pollen onto stigmas of *Aloe reitzii* var. *reitzii*. However, bird-excluded treatments produced significantly lower seed set compared to treatments that included all visitors ($P < 0.001$), showing *Aloe reitzii* var. *reitzii* is predominantly bird-pollinated and bees probably play only a minor role in its pollination. Cape Weavers (*Ploceus capensis*) and Malachite Sunbirds (*Nectarinia famosa*) are both effective pollinators of *Aloe reitzii* var. *reitzii*, as evidenced by the comparably high pollen loads observed on both species ($P > 0.05$). *Aloe reitzii* var. *reitzii* flowers produce copious dilute nectar, characteristic of a generalist bird pollination syndrome. The long floral tubes and generalist nectar properties of *Aloe reitzii* var. *reitzii* suggest that floral traits are intermediate between a generalist and specialist bird pollination syndrome, enabling effective pollination by both specialist and generalist nectarivores. Additionally, effective cross-pollen transfer by both bird species is facilitated by the strongly exserted style and filaments of *Aloe reitzii* var. *reitzii*, coupled with each species’ feeding behaviour. Therefore, floral characteristics and bill morphology are not always indicative of the most effective bird pollinators of aloes. Although *Aloe reitzii* var. *reitzii* is effectively pollinated by Malachite Sunbirds and Cape Weavers, the consistent abundance and frequent visitation of Cape Weavers to *Aloe reitzii* var. *reitzii* flowers in multiple years, indicate they are the primary pollinators of *Aloe reitzii* var. *reitzii.*

KEYWORDS

Bees; Cape Weaver; Malachite Sunbird; Nectarivore; Ornithophilous

INTRODUCTION

According to the pollination syndrome hypothesis, floral traits associated with attracting particular pollen vectors are used to infer likely pollinators (Fenster *et al.*, 2004). Based on this hypothesis, particular pollinators can be grouped together because they exert similar selection pressures on plant traits, resulting in a suite of convergent floral traits in unrelated plants associated with specific pollinator guilds (Johnson and Steiner, 2000; Pauw, 2006; Ollerton *et al.*, 2009). Hence, pollinators are considered to be strong selective agents, and many plant traits are considered to be adaptations to specific pollinators (Stebbins, 1970; Johnson and Steiner, 2000; Fenster *et al.,* 2004). Flowering season and floral traits, such as morphology (colour, size and shape of perianth) and rewards (e.g., nectar, oils) are therefore said to be a reflection of the most effective pollinator ("most effective pollinator syndrome"; Stebbins, 1970; p. 318), reflecting foraging preferences and morphology of their pollinators (Fægri and van der Pijl, 1979; Ollerton *et al.*, 2009).

Various studies have proven the validity of the pollination syndrome hypothesis (e.g., Johnson and Steiner, 2000; Fenster *et al.*, 2004; Rosas-Guerro *et al.*, 2014), indicating that there is a strong association between certain floral traits and functional groups of pollinators (Fenster *et al.*, 2004), and that these convergent traits are adaptations to the most effective pollinator (Rosas-Guerro *et al.*, 2014). However, the pollination syndrome hypothesis cannot account for the vast diversity of floral phenotypes observed today, nor can it be used to predict the primary pollinators of most plant species (Ollerton *et al*., 2009). Additionally, it is often difficult to distinguish which animals serve as pollinators or just visitors (Fægri and van der Pijl, 1979). Therefore, the reliability of pollination syndromes in predicting primary pollinators has been questioned (Waser *et al.*, 1996; Smith *et al.*, 2008; Ollerton *et al.*, 2009), and using convergent floral traits to determine the most effective pollinators should be used with caution (Ollerton *et al.*, 2009).

Evolutionary shifts to bird pollination have resulted in morphological changes in many floral features, particularly those associated with the attraction of birds, deterrence of unwanted flower visitors, protection from foraging by nectar-thieving birds, and accurate placement of pollen on birds' bodies (Cronk and Ojeda, 2008). Floral adaptation to birds therefore includes a broad syndrome of convergent floral traits, such as vivid red/orange floral displays (also associated with bee deterrence; Rodríguez-Gironés and Santamaria, 2004; Cronk and Ojeda, 2008), long narrow floral tubes, odourless flowers (Fægri and van der Pijl, 1979; Ollerton, 1996; Proctor *et al.*, 1996), and commonly, the production of copious dilute nectar, which serves as the main pollinator reward (Stiles, 1976; Cronk and Ojeda, 2008; Johnson and Nicolson, 2008).

Bird-pollinated plants either have specialized or generalized pollination systems, based on whether they are pollinated by specialist nectarivores (e.g., sunbirds and sugarbirds) or generalist nectarivores (e.g., weavers, bulbuls and orioles, etc.), respectively. These pollination systems are mainly distinguished by differences in nectar traits, namely nectar volume, concentration and sucrose content (Johnson and Nicolson, 2008), as well as differences in corolla morphology (Feinsinger and Colwell, 1978). Corolla length and curvature can be used to distinguish between specialist and generalist pollination systems, as these differences affect the extraction efficiencies of birds due to bill morphology and feeding positions (Stiles, 1981; Botes *et al.*, 2008; Johnson *et al.*, 2020), thus strongly influencing their flower choice (Stiles, 1978). Plants pollinated by specialist nectarivores are characterized by highly concentrated nectar (~15 – 25% w/w), with a relatively low nectar volume (~10 – 30 µl) and high sucrose content (~40 – 60%; Johnson and Nicolson, 2008). In comparison, plants pollinated by generalist nectarivores have dilute nectar (~8 – 12% w/w), larger nectar volumes (~40 – 100 µl), and a low sucrose content (0 – 5% w/w; Johnson and Nicolson, 2008). However, the copious nectar supply provided by both specialized and generalized bird-pollinated flowers, also attracts other insects, particularly bees, which may or may not play a significant role in the plants' pollination.

Even though plants with a bird-pollination syndrome are pollinated by specialist and generalist birds, other floral visitors, particularly insects, may play a significant role. For example, *Protea caffra* Meisn. capitula, which mainly display ornithophilous traits, are visited by both birds and insects, where insects are also shown to be effective agents of cross-pollination (Steenhuisen *et al.*, 2012). "Bird-adapted" flowers may therefore rely on insects for pollination, even though these insects may not be the primary pollinators. Hence, additional floral visitors may serve as secondary pollinators or co-pollinators of many "bird-pollinated" plants (Botes *et al.*, 2009a; Cousins *et al.*, 2013).

Birds are considered more reliable pollinators compared to insects, as they are more constant visitors, especially in unpredictable climates and flowering seasons, such as the dry winter months, when many bird-pollinated plants flower (Fægri and van der Pijl, 1979). Since birds are homeothermic and have high energy requirements, they still need to continue to forage during winter, whereas insects are able to reduce their metabolism and remain inactive (Stiles, 1978). Therefore, the copious nectar produced by many South African winter-flowering plants, such as *Aloe* L. and *Protea* L. species, serve as key food sources to meet the high energy demands of birds (Hoffman, 1988; Hargreaves *et al.*, 2004; Botes *et al.*, 2009a).

Many South African aloes flower in winter, producing high quantities of nectar, which serve as a major food resource for both birds and insects (Symes and Nicolson, 2008; Forbes *et al.*, 2009; Cousins and Witkowski, 2012). Since aloes are an important food source for a variety of avian communities, they have been shown to shape and drive seasonal changes in bird communities (e.g., *Aloe marlothii* A.Berger, Symes *et al.*, 2008; and *Aloe ferox* Mill., Forbes *et al.*, 2009; Kuiper *et al.*, 2015). Birds are therefore key role players in the pollination system of many South African aloes (Stokes and Yeaton, 1995; Botes *et al.*, 2009a; Arena *et al.*, 2013), most of which are characterized by the bird pollination syndrome (e.g., *A. africana* Mill., *A. speciosa* Baker, Botes *et al.*, 2008, 2009a; *A. marlothii*, Symes and Nicolson, 2008; Symes *et al.*, 2009; Hargreaves *et al.*, 2012).

The floral traits of ornithophilous aloes, namely corolla tube length and nectar properties, vary according to whether they are pollinated by specialist or generalist nectarivores (Botes *et al.*, 2009a; Hargreaves *et al.*, 2012). Aloes pollinated by long-billed specialist nectarivores, are mainly characterized by long-tubed flowers (30.3 – 32.1 mm), with small volumes of relatively concentrated nectar (19.4 – 39.6 µL; 12.1 – 22.1 % w/w), such as *A. arborescens* Mill., *A. boylei* Baker. and *A. maculata* Medic. (Hargreaves *et al.*, 2012). In comparison, aloes pollinated by short-billed nectarivores — such as *A. ferox*, *A. marlothii* and *A. vryheidensis* Groenew, have mid-length (23.5 – 25.5 mm) to short-tubed flowers (7.8 – 14.3 mm), with larger volumes of relatively dilute nectar (41.1 – 81.3 µL; 8.3 – 11.6 % w/w; Botes *et al.*, 2009a; Hargreaves *et al.*, 2010, 2012). However, even though many aloes are primarily bird-pollinated (Symes *et al.*, 2008; Botes *et al.*, 2009b; Arena *et al.*, 2013; Cousins *et al.*, 2013; Kuiper *et al.*, 2015; Patrick *et al.*, 2018), small non-flying mammals (e.g., mice and sengi; Payne *et al.*, 2016) and insects (Van Wyk and Smith, 2008; Hargreaves *et al.*, 2008; Symes *et* *al.*, 2009; Wilson *et al.*, 2009; Duffy *et al.*, 2014), have also been confirmed as pollinators for some aloes.

Floral traits characteristic of the bird-pollination syndrome in aloes, such as their exserted stamens, abundance of pollen, and the production of high quantities of nectar, can make them attractive to insects, especially bees (Botes *et al.*, 2009a). Solitary bees (e.g., *Lassioglossum* sp. and *Amegilla* sp.), colonial bee species (e.g., allodapine bee species) and sub-social bees (*Apis mellifera*) are particularly known to visit aloe flowers (Hoffman, 1988; Botes *et al.*, 2009b; Patrick *et al.*, 2018). Additionally, some aloes also have greenish-yellow flowers (e.g., *A. linearifolia* A.Berger, Botes *et al.*, 2009b) and white-greenish flowers (e.g., *A. inconspicua* Plowes, Hargreaves *et al.*, 2012), which are commonly associated with insect pollinators, such as bees and beetles (Fægri and van der Pijl, 1979). Bees are the primary pollinators of summer-flowering aloes, which have floral traits consistent with insect pollinators (e.g., *A. linearifolia*, *A. minima* Baker., Botes *et al.*, 2009b; *A. inconspicua*, Hargreaves *et al.*, 2008, 2012). In some ornithophilous *Aloe* species, bees are either primary pollinators (e.g., *A. dominella* Reynolds, Hargreaves *et al.*, 2012; *A. greatheadii* var. *davyana* (Schönland) Glen & D.S.Hardy, Human and Nicolson, 2006, 2008; Symes *et al.*, 2009; and *A. linearifolia*, Botes *et al.*, 2009b) or play a secondary role to bird pollinators (e.g., *A. arborescens*, Hargreaves *et al.*, 2012; *A. ferox*; Botes *et al.*, 2008, 2009a; Hargreaves *et al.*, 2012; Kuiper *et al.*, 2015; *A. pruinosa* Reynolds, Wilson *et al.*, 2009). Therefore, despite having many floral traits characteristic of the bird pollination syndrome, both birds and insects, primarily bees, have been shown to play various roles in the pollination system of ornithophilous *Aloe* species.

Similar to most South African aloes, the summer-flowering *Aloe reitzii* Reynolds var. *reitzii* (hereafter referred to as *A. reitzii*) has floral traits consistent with the bird pollination syndrome, displayed by their red/orange long tubular perianths (Van Wyk and Smith, 2008) and copious nectar production (Symes, 2017; Payne, 2019). However, both birds and bees have been observed as frequent visitors to *A. reitzii*, but due to the number of birds observed, birds have been suggested to play a more significant role in its pollination biology (Symes, 2017; Payne, 2019). Even though insects, such as grasshoppers and beetles, were common visitors, it was suggested that they play no role in the pollination of *A. reitzii,* as they were mostly been observed eating floral parts (Payne, 2019). However, since honeybees were observed drinking nectar around the entrance of flowers (the dilute nectar of *A. reitzii* runs out of flowers such that bees would not need to enter flowers to reach nectar; Payne, 2019), it is possible that bees play a significant role in the aloe’s reproductive biology. Therefore, the role of bees in the pollination system of *A. reitzii* needs to be assessed.

Aloe reitzii is commonly visited by both specialist (e.g., Malachite sunbird *Nectarinia famosa*) and generalist nectarivores (e.g., Cape Weaver *Ploceus capensis*; Symes 2017; Payne, 2019). However, it is unclear whether *A. reitzii* has a specialist or generalist bird pollination syndrome, as two previous studies, during February of 2014 (Symes, 2017) and 2017 (Payne, 2019), different patterns of visitation rates between these bird species were observed and each study yielded contrasting nectar properties. Firstly, in both studies generalists (Cape Weaver) were abundant visitors to *A. reitzii*, contributing 60.4% (2014; Symes, 2017) and 41% (2017; Payne, 2019) of all bird visits. However, the abundance of specialist bird species (Malachite Sunbird) fluctuated, contributing only 27% of bird visits in 2014 (Symes, 2017), compared to being the predominant bird visitor in 2017 (49% of visits; Payne, 2019). Secondly, the nectar properties observed by Symes (2017) in 2014 suggested that *A. reitzii* is likely pollinated by specialist birds (volume: 36 ± 27 μL, concentration: 16.5 ± 1.7 % w/w), whereas the nectar properties reflected generalist preferences in February 2017 (volume: 46.5 ± 11.72 μL, concentration: 10.7 ± 0.5 % w/w) in 2017 (S. Payne, unpubl. data). Due to these contrasting results, it is important to determine whether generalist or specialist nectarivores are the primary pollinators of *A. reitzii*.

This study therefore aimed to investigate the role of birds and insects, particularly bees, in the reproductive biology of *A. reitzii*, and the relative importance of generalists and specialist birds in its pollination. The pollinator effectiveness of birds and bees in *A. reitzii* was investigated, by comparing the visitation rates of specialist and generalist nectar feeding birds, and the pollen loads of birds and insects. To determine the role of bees in the pollination system of *A. reitzii,* pollinator exclusion experiments were set up, and differences in fruit set and seed set between treatments were compared, and pollen load of birds and bees was compared. The relative importance of specialist and generalist nectarivore birds was determined by investigating nectar properties of *A. reitzii*, and the visitation rates, pollen load and main pollen deposition sites of these pollinator guilds. To determine whether generalists or specialist nectarivores could have contacted the stigmas of flowers visited, their bill morphology was compared to the floral morphology of *A. reitzii.*

MATERIALS AND METHOD

Study species and study site

Aloe reitzii is endemic to South Africa, with localised distributions in Mpumalanga, and restricted distributions in Limpopo and Gauteng (Van Wyk and Smith, 2008). *Aloe reitzii* flowers between February and March, i.e. in summer. It is characterized by long (~30 – 50 mm; Table 2, Chapter 3) red-orange tubular flowers that turn yellow as they mature (Figure 3.1A; Van Wyk and Smith, 2008). The study was conducted on Klipbankspruit Farm (25° 16′ 21.9″ S, 30° 3’ 14″ E, ~1840 m.a.s.l.), approximately 30 km north-west of Dullstroom where *A. reitzii* is abundant.

Floral visitors

Observations of floral visitors were conducted to identify: i) the main floral visitors, and ii) visitation rates of floral visitors of *Aloe reitzii*. To record visitors to flowering aloes, motion-sensitive camera traps (Bushnell HD Model No. 119537 and Bushnell Natureview Model No. 119740, China) were set up for 18 randomly selected aloes. Camera traps were placed on tripods (height ~ 1.5 m), at least 1 m away from each flowering aloe, facing southwards to minimize the glare from the sun (as per Symes, 2017). Cameras were set at high sensitivity to take three photographs when motion sensors were activated, with 3 s intervals between activations, and left to run for 24 hours, throughout a three week period during peak flowering (Symes, 2017). Memory cards were downloaded daily and batteries changed as necessary. To calculate the visitation rates for any floral visitors, including bees, the total time spent visiting *A. reitzii* was calculated using the time stamp of the first and last photograph of each visitor, on camera traps for all floral visitors. Additionally, incidental observations of floral visitors were conducted for ~five hours daily for the three-week study period. These observations were conducted across the study area (~1.5 km^2), which comprised more than 600 mature and flowering aloes (Payne, 2019). An animal was recorded as a visitor if it touched the aloe inflorescence, for example, by probing the flowers or perching on the raceme (Payne *et al.*, 2016). To calculate the hourly visitation rate, the visitation rates for floral visitors were calculated each hour over a 24-hour period, for the sampling period (mean number of visits/plant/hour; Symes, 2017).

Insect visitors to *A. reitzii* were collected and swabbed for pollen to determine whether visitors to aloes were legitimate pollinators or just floral visitors. To determine the role of insects in the pollination system of *A. reitzii*, insect visitors were caught using insect nets, and vials were used to catch any insects crawling into flowers. Approximately five to 10 individuals of each invertebrate species were caught where possible. To avoid moisture on the insect, which may result in pollen removal, caught insects were frozen until examination. Insects were placed on a microscope slide and rinsed with 90% ethanol to remove pollen, and once the ethanol had evaporated, the residue was stained with Calberla's solution (Knox *et al.*, 1985). Pollen was examined under a light microscope at 100X magnification, and number of pollen grains was counted using a grid transect placed over each microscope slide.

To determine which bird species carry pollen of *Aloe reitzii*, and whether generalist or specialist pollinators play a more important role in its pollination, birds were caught using mist-nets by a licensed bird-ringer during peak flowering season (ringer no.736, permit no. 0926), and swabbed for pollen. Mist nets were set up across the study site for five days. Eight nets were set up across the study site, each net spanning 12 x 3 m. According to the observed movement of birds, three nets were moved to different locations to increase the likelihood of catching birds in the nets; the other five remained in their original positions. The nets were checked at hourly intervals, or more frequently, depending on the weather. The nets were closed in the late afternoon and opened early in the morning, consistent with bird activity. Sampled birds were ringed to avoid re-sampling the same individual.

The main site of pollen deposition was determined by swabbing the throat, beak, crown and foot of each bird, each with a separate piece of fuschin gel (Beattie, 1971). After swabbing, each bird was photographed for bill curvature measurements, and then released on site. The fuschin gel with pollen was melted onto slides and permanent slides were produced on site. Using a light microscope under 100X magnification, the pollen grains were counted using transects on each slide. The total pollen load was estimated by combining the pollen scores of the deposition sites for each individual (Anderson, 2003). A reference pollen collection was created from randomly selected flowering *Aloe reitzii* and *Leonotis leonurus* (L.) R.Br., as these were the main plants visited by the sunbirds. Mostly *Aloe* and *Leonotis* pollen was observed, so pollen was classified as "Aloe", "Leonotis" or "Other". This enabled me to determine if *Aloe reitzii* pollen was present on each bird species.

Pollinator effectiveness of birds and bees

Pollinator exclusion experiments were used to determine the relative contributions of birds and insects to the pollination and reproduction of *Aloe reitzii*. Individual racemes on twenty-four aloes were randomly assigned to one of three treatments: open, bird exclusion, and total exclusion (Figure 3.1B), and their location was recorded using a handheld GPS. The open treatment allowed access to all floral visitors (birds and insects) and was left uncovered. The bird exclusion treatment only allowed access by insects visitors, and was achieved by placing a plastic mesh cage (20 x 15 mm^2 aperture) around the inflorescences, allowing access by bees and other insects (Figure 3.1B). Total exclusion was effected by placing a fine organza bag over each raceme (Figure 3.1B; Arena *et al.*, 2013; Cousins *et al.*, 2013).

Individual racemes were covered before anthesis (early February) until the end of the flowering season (late March), when racemes were harvested and the number of fruits on each raceme was counted. Fruits were collected four weeks after the peak flowering season (Figure 3.1C). To calculate fruit set, the percentage of flowers per raceme that produced fruit was determined. The number of pedicels present after flowers had fallen off enabled accurate counting of flowers on the raceme (Symes *et al.*, 2009). Young flowers that did not mature were excluded from fruit set calculations. Seed set was determined by first air drying each individual fruit (10 fruit/treatment raceme) at ambient temperature. Once the fruit had dehisced, the number of seeds in each fruit were counted to calculate the average number of seeds per fruit (Arena *et al.*, 2013). To calculate total seed production, the average number of seeds per fruit was multiplied by total fruit production for each plant (Arena *et al.*, 2013).

Bird pollination system

Nectar properties

To determine whether the nectar properties of *Aloe reitzii* fall within a generalist or specialist bird pollination system, nectar volume and concentration were measured across eleven aloes with ≥2 flowering racemes, during peak flowering. On each aloe, one raceme was bagged with a fine organza bag the day before extractions to determine nectar availability (screened), and nectar standing crop (unscreened) was determined by extracting nectar from an open raceme. Nectar was extracted during the day using disposable haematocrit tubes (50 µL) from one flower per raceme for each treatment (screened and unscreened) every two hours, starting from 07h00 to 17h00 (as per Payne, 2019). Sampling was destructive. To record temperature and humidity during nectar sampling, temperature and humidity data loggers

(Thermocron iButtons, Australia) were placed on five randomly selected sampled plants. Nectar volume was recorded, and nectar sugar concentration (% w/w) was measured using a MISCO Palm Abbe digital refractometer (Model no. PA201). Only fully-open flowers, with anthers newly dehisced were sampled, as nectar is most abundant during this stage in the aloe's floral development ("Stage 2 flowers", Symes and Nicolson, 2008; Payne, 2019).

Primary bird pollinator

Since the Malachite Sunbird (*Nectarinia famosa*) and Cape Weaver (*Ploceus capensis*) were observed as main visitors in previous studies (Symes, 2017; Payne, 2019), sites of pollen deposition were noted and the pollen loads on each of these species were measured, as outlined above, to determine which species is the more important pollinators of *A. reitzii*.

The bill length of the Malachite Sunbirds and Cape Weavers, as well as selected floral characteristics were measured and compared to determine whether either or both bird species could have contacted the stigmas of flowers visited. The bill lengths (measured from the union of the bill with the base of the skull to the tip of the bill) of Malachite Sunbirds and Cape Weavers caught in mist nets were measured, and a mean was calculated for each species. In addition, the bill length measurements for these two species were extracted from datasets of previously ringed birds on the Safring Database (Safring, 2020). To determine whether the bills of each bird species can fit into the flower, perianth tube length of 30 randomly selected flowers on individual racemes was measured from the base to apex of flower and compared to the bill length of each bird species. To determine whether flowers may be penetrated by Malachite Sunbird and Cape Weaver beaks, the curvature of the culmen of the beaks was measured and compared to measurements of corolla curvature. Stamen length and style length of the 30 randomly selected flowers were measured to determine whether the bills and other sites of pollen deposition of each species came into contact with reproductive organs of *A. reitzii* flowers. Bill length measurements were conducted using a non- digital calliper; bill curvature, perianth tube length and corolla curvature were measured from photographs, using ImageJ (Schneider *et al.*, 2012). A ruler was included in the pictures of the birds and flowers and was used to scale images on ImageJ. The likelihood of pollen transfer by each bird species was determined by comparing the bill length and bill curvature of the birds and the measured morphological floral characteristics.

Data analysis

Floral visitors

To test for differences in the hourly visitation rate of birds, the visitation rate for each pollinator guild (specialist vs. generalist) was modelled as count data, using a Generalized Linear Model (GLM), with time and guild as predictor variables and an offset for hours of sampling effort (i.e. number of camera trap hours), with Poisson distribution and log-link function (as per Payne, 2019). Time was converted to radians to standardize daylight and night-time hours. A GLM was used to test for differences in total hourly visitation rates of bird species, with time and species as predictor variables, with an offset for hours of sampling effort, and a Poisson distribution. To compare overall visitation rates by Cape Weavers and Malachite Sunbirds, a Wilcoxon Rank Sum Test was used.

Differences in total pollen load among bird guilds were tested using a GLM, with species and sex as predictor variables, with a Gaussian distribution. A two-sample t-test assuming equal variance was used to compare total pollen load between Cape Weavers and Malachite Sunbirds. Total pollen load was log-transformed to follow a normal distribution. To test for differences in pollen load on different pollen deposition sites of birds, pollen load was modelled using a GLM, with species and pollen deposition site as predictor variables, with Poisson distribution. Differences among pollen deposition sites in Cape Weavers, and in Malachite Sunbirds, were tested using two separate ANOVAs, and a Tukey's Honest Significant Difference (HSD) *post-hoc* analysis was used to determine which pollen deposition sites were significantly different. Total pollen load and pollen load per deposition site on each bird species was log-transformed to follow a normal distribution.

A Kruskal-Wallis test was used to test for differences in pollen load among insect guilds (insect functional groups, e.g., bees, flies, wasps, beetles etc.) and insect species, as data could not be transformed to follow a normal distribution, and a Kruskal-Wallis Multiple Comparison *Post-hoc* test was used to determine where the differences lie. No pollen was present on Halictinae bee species, so a two-sample t-test, assuming unequal variance, was used to compare pollen load between honeybees and the sweat bee *Lasioglossum* sp. Pollen load was log-transformed to follow a normal distribution. Differences in total pollen load among bee and bird species were determined using an ANOVA, and a TukeyHSD *post-hoc* test was used to determine if there were differences among species.

Pollinator effectiveness of birds and bees

For pollinator exclusion experiments, differences in fruit set, seed set and total seed production for each treatment were each determined using a Kruskal-Wallis test. A Kruskal-Wallis Multiple Comparison *Post-hoc* test was used to determine if there were significant differences among treatments.

Bird pollination system

Nectar volume and concentration were modelled using a Generalized Linear Mixed-Effects Model (GLMM), with time, treatment, temperature as predictor variables, and individual plants as a random effect, with a Poisson distribution. Time was converted to radians to standardize daylight hours. A simple linear regression was used to determine if nectar volume and concentration were correlated with temperature and humidity.

To test for differences in bill morphology of bird species, bill length and bill curvature were modelled using a GLM, with species and sex as predictor variables, with a Gaussion distribution. To test for a correlation between bill length of Cape Weaver and Malachite Sunbird, with corolla tube length, stamen length and style length of *A. reitzii,* a simple linear regression was used. A simple linear regression was also used to test if there is a correlation between bill curvature of Malachite Sunbird and Cape Weaver and corolla tube curvature of *A. reitzii.* All GLMMs were run using the R package ‘lme4’ (Bates *et al*., 2015) and Kruskal-Wallis Multiple Comparison *Post-hoc* tests were run using the R package ‘pgirmess’ (Giraudoux *et al*., 2018). All statistical analyses were performed in R (v.4.0.0.; R Core Team, 2020).

RESULTS

Floral Visitors

Visitor Observations

Birds. Camera trap observations of all bird visitors were conducted to identify the main floral visitors of *A. reitzii* and their visitation rates. A total of ten bird species (three specialists and seven generalists) were recorded visiting *A. reitzii* during the sampling period (*N* = 1132 visits, 4633 camera hours, Table 3.1). Only two birds could not be identified to species level, due to poor lighting, but were identified as generalist bird species based on their larger body size and shape of their bills.

The Cape Weaver (*Ploceus capensis*) was the most common generalist and overall visitor, accounting for 57.5% of all bird visits (Figure 3.1D–F, Table 3.1). The Malachite Sunbird (*Nectarinia famosa*) was the most common specialist visitor, accounting for 33.3% of all bird visits (Figure 3.1G–H, Table 1). Both Cape Weavers and Malachite Sunbirds were recorded on 17 of the 19 aloes observed (along with other bird species), and only one of the sampled aloes was solely visited by Cape Weavers.

Plants were visited by birds throughout the day until the evening (daylight hours were from ~05h40 to ~18h40; Thorsen, 2021), with Cape Weaver as the earliest visitor to the aloes at 05h13. Visitation to the aloes by generalists peaked at 06h00, with specialist visitation only starting to increase from 07h00, and with visitation by both guilds decreasing from 12h00 onwards (Figure 3.2). However, there was a second increase in visitation by sunbirds at 17h00 (Figure 3.2). Cape Weavers were the most frequent visitors from 05h00 to 08h00 and remained regular visitors throughout the day from 09h00 onwards (Figure 3.2, Figure 3.3A). Malachite Sunbirds were the most frequent visitor from 12h00 – 19h00, and similar to Cape Weavers, were consistent visitors throughout the day (Figure 3.2, Figure 3.3B). Visits by Malachite Sunbirds peaked at 12h00 and 17h00. Only Cape Weavers ($n = 2$) and Malachite Sunbirds ($n = 8$) visited the aloes between 18h01 and 18h48, with the latest visit made by an unknown generalist nectarivore at 19h36. Male Malachite Sunbirds ($N = 382$ visits) visited flowers more frequently than female Malachite Sunbirds ($N = 12$ visits), whereas both male ($N = 344$ visits) and female ($N = 339$ visits) Cape Weavers were frequent visitors to *A. reitzii*.

Generalist and specialist nectar-feeding bird visitation to aloes differed significantly ($\beta = -0.42 \pm 0.06$, $P < 0.001$), as well as visitation time periods for each guild ($\beta = -0.27 \pm 0.05$, $P < 0.001$), as generalists dominated visitation in the early morning (Figure 3.2). Overall bird visitation rates did not differ throughout the day ($\beta = 0.124 \pm 0.09$, $P = 0.16$), as specialists were regular visitors during the course of the day, from early morning to early evening (Figure 3.2). Cape Weaver visitation rates were significantly different compared to other bird species ($\beta = 0.56 \pm 0.095$, $P < 0.001$), and were affected by time ($\beta = 0.54 \pm 0.15$, $P < 0.001$), as visitation by Cape Weavers peaked in the early morning at 06h00 and declined thereafter. Cape Weaver and Malachite Sunbird bird visitation rates were significantly different ($U = 10486$, P

< 0.05). Overall visitation rate by Cape Weavers changed over time ($\beta = 0.54 \pm 0.15$, $P < 0.001$), whereas the visitation rate of Malachite Sunbirds did not ($\beta = -0.029 + 0.15$, $P = 0.85$), as Cape Weavers dominated visitation in the early morning, while Malachite Sunbirds dominated visits from midday onwards.

The overall mean hourly visitation rate by all birds was 0.28 ± 0.03 visits/plant/hour. Cape Weaver, Malachite Sunbird and Amethyst Sunbird (*Chalcomitra amethystina*; Figure 3.1F) visitation rates were 0.36 ± 0.037, 0.23 ± 0.033 and 0.11 ± 0.014 visits/plant/hour respectively. These species were all observed probing flowers for nectar by perching below (Figure 3.1D; Figure 3.1G) or above the open flowers (Figure 3.1F). Greater Double Collared Sunbirds (*Cinnyris afer*) were not recorded on camera traps but were also observed removing nectar (J. Minnaar, *pers. obs.*; Figure 3.1 J–K.). The hourly overall visitation rate by Cape Weavers was significantly higher than that of the Malachite Sunbirds ($t = -3.03$, $df = 32$, $P < 0.01$).

Figure 3.1. Pollination treatments of, and bird visitors to, *Aloe reitzii* var. *reitzii*: A) Mature, flowering *Aloe reitzii* var. *reitzii* plant; B) Pollinator exclusion experiment on an *Aloe reitzii* var. *reitzii* plant, from left to right: Total Exclusion (Bagged), Bird Exclusion (Caged) and All Pollinators (Open); C) Fruiting *Aloe reitzii* var. *reitzii* raceme; D) Male Cape Weaver (*Ploceus capensis*) feeding on nectar; E) Female (left) and Male (right) Cape Weaver perched; F) Male Cape Weaver feeding ; G) Male Malachite Sunbird (*Nectarinia famosa*) feeding on nectar; H) Male Malachite Sunbird perched; I) Male Amethyst Sunbird (*Chalcomitra amethystina*) perched; J) Male Greater Double-Collared Sunbird (*Cinnyris afer*) feeding on nectar; K) Male Greater Double-Collared Sunbird perched. Scale bars = 10 cm. Photographs A – C and I – K by Jessica Minnaar. Photographs D – H are camera trap photos.

Table 3.1. Percentage contribution (%) to visitation and mean visitation rates (visits/hour/plant; mean ± SE) of all specialist and generalist nectarivore bird species recorded by camera traps on *Aloe reitzii* var. *reitzii* plants ($N = 19$) during the February to March 2020 flowering season. Percentage contributions were calculated as percentage of the total number of visits by that species. The activity of each bird species was noted as "feeding only", "perching only" or "both". Only researcher (incidental) observations were made for Greater Double-Collared Sunbird, and two visits of an unknown generalist were recorded on camera traps.

Order	**Family**	**Species**	**Contribution**	**Visitation rate**	**Activity (Feeding/Perching)**	**Researcher or Camera observation**
SPECIALIST NECTARIVORES						
Passeriformes	Nectariniidae	Amethyst Sunbird *Chalcomitra amethystina*	2.47	0.11 ± 0.01	Feeding and perching	Camera only
		Greater Double-Collared Sunbird *Cinnyris afer*	-	-	Feeding	Researcher only
		Malachite Sunbird *Nectarinia famosa*	33.3	0.23 ± 0.33	Feeding and perching	Researcher and camera
Total number of specialist visits				405		
Total specialist rate				0.07 ± 0.02		
GENERALIST NECTARIVORES						
Passeriformes	Laniidae	Common Fiscal *Lanius collaris*	3.89	0.11 ± 0.01	Perching only	Researcher and camera
	Malaconotidae	Bokmakierie *Telophorus zeylonus*	0.27	0.08 ± 0.002	Perching only	Camera only
	Motacillidae	Cape Longclaw *Macronyx capensis*	0.09	-	Perching only	Camera only
	Muscicapidae	Buff-Streaked Chat *Campicoloides bifasciata*	0.71	0.09 ± 0.005	Perching only	Camera only
	Ploceidae	Cape Weaver *Ploceus capensis*	57.5	0.36 ± 0.04	Feeding and perching	Researcher and camera

Order	Family	Species	Contribution	Visitation rate	Activity (Feeding/Perching)	Researcher or Camera observations
GENERALIST NECTARIVORES						
Passeriformes	Pycnonotidae	Dark-Capped Bulbul *Pycnonotus tricolour*	1.5	0.17 ± 0.03	Feeding and perching	Researcher and camera
	Unknown	Unknown	0.27	0.083	Perching only	Camera only
Total number of generalist visits				727		
Total generalist rate				0.27 ± 0.05		

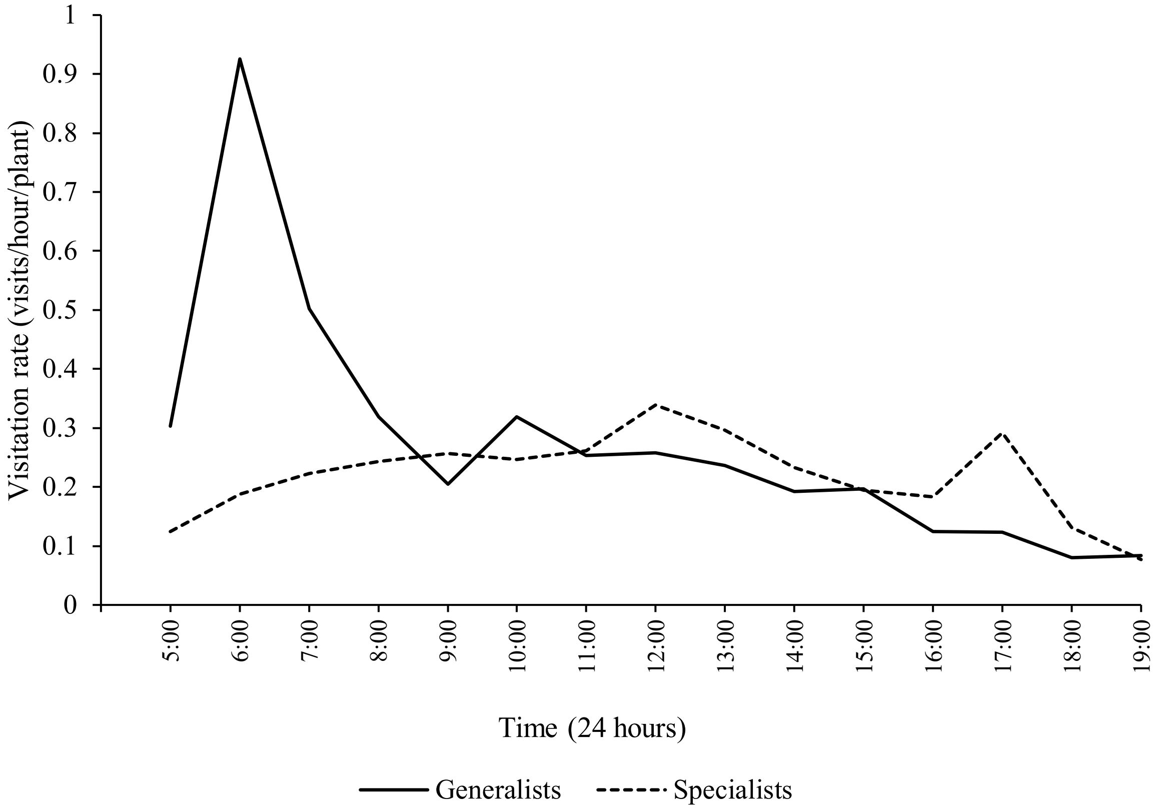

Figure 3.2. Mean hourly visitation rates (no. visits/hour/ plant) for generalist (solid line) and specialist (dotted line) nectarivores on *Aloe reitzii* var. *reitzii* plants during the February to March 2020 flowering season (n = 19 plants, 4633 camera hours), during daylight hours (05h00 – 19h00).

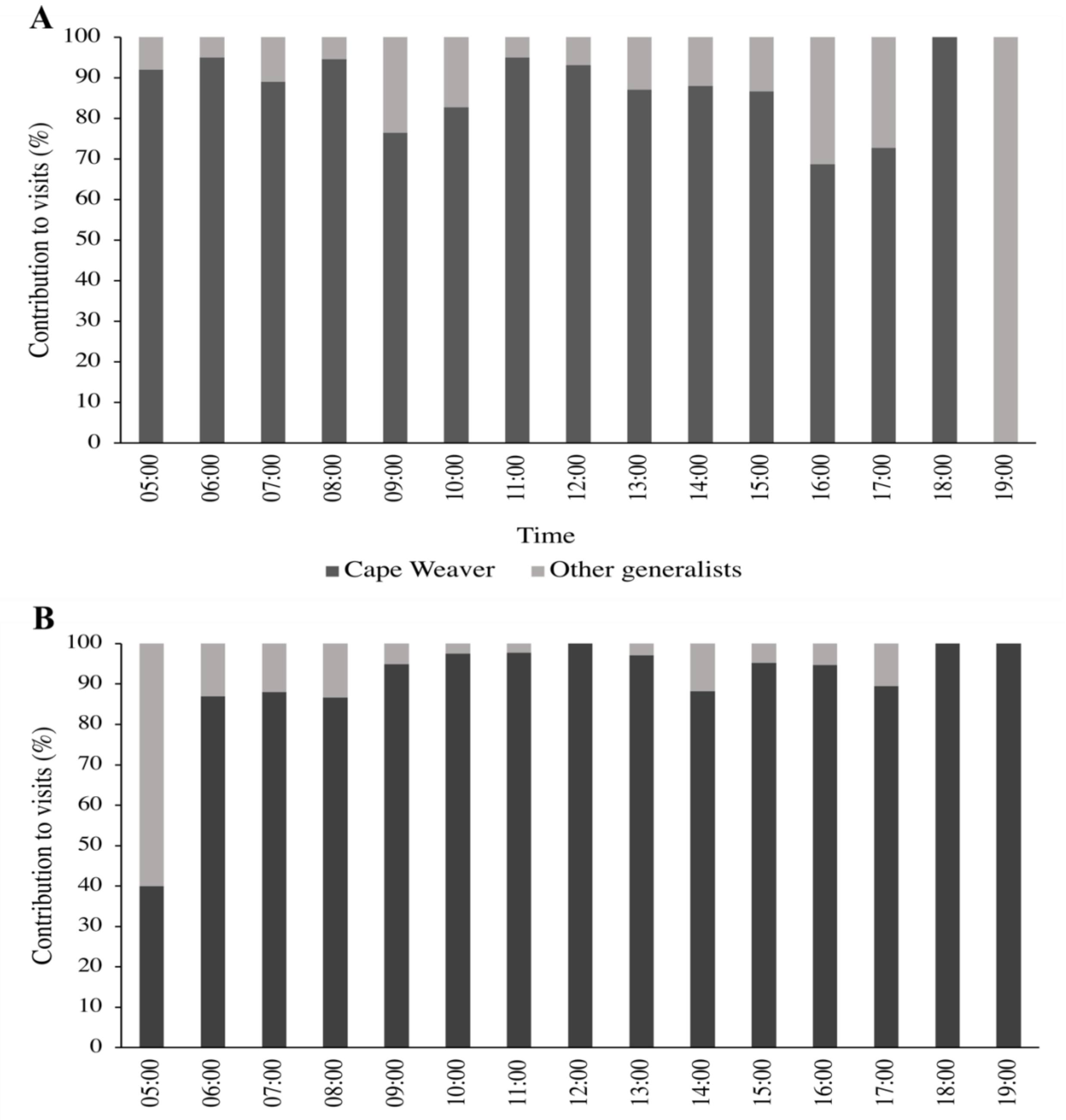

Figure 3.3. Percentage contribution of bird species to visitation at each daylight hour (05:00 – 19:00) to *Aloe reitzii* var. *reitzii* from camera trap observations, by A) Generalist nectarivores (n = 7 species Cape Weaver *Ploceus capensis* = dark grey, other generalist species = light grey) with single unknown generalist bird species observed at 06:28 and 19:36; and B) Specialist nectarivores (n = 2 species; Malachite Sunbird *Nectarinia famosa* = dark grey, Amethyst Sunbird *Chalcomitra amethystina* = light grey), during February – March 2020 flowering season.

Insects. Observations of insect floral visitors were conducted to identify the main insect floral visitors to *A. reitzii* flowers. Approximately thirty invertebrate species were observed visiting flowers of *A. reitzii* (Table 3.2). Honeybees (*Apis mellifera*) were the most frequent invertebrate visiting flowers during daylight hours from 05h00 to 19h00 (n = 3357 camera trap records from seven aloes), contributing approximately 85% of all insect visits to *A. reitzii*. They were observed removing nectar and pollen (Figure 3.4A). Solitary bees (*Lasioglossum* sp.) were also frequent visitors, often observed crawling around on *A. reitzii* flowers, drinking nectar. Elegant Grasshoppers (*Zonocerus elegans elegans*; Figure 3.4B), Toxic Milkweed Grasshoppers (*Maura rubroornata*; Figure 3.4C) and Koppie Foam Grasshoppers (*Dictyophorus spumans*; Figure 3.4D), were recorded perching on racemes, and were often observed eating the flower buds at the tips of the racemes, moving between racemes and plants. Various wasp species, such as *Hylaeus* sp. (Figure 3.4E), were observed crawling around *A. reitzii* flowers, and were often observed entering flowers. Numerous fly species, such as *Sarcophagidae* sp., were observed sitting on racemes (Figure 3.4F). Coleoptera, namely *Dischista rufa* and *Harmonia* sp., were observed perching on racemes, or nestled between individual flowers. African Migrant (*Catopsila florella;* Figure 3.4G), Broad-bordered Grass Yellow (*Eurema brigitta*) and Citrus Swallowtail (*Papilio demodocus*; Figure 3.4G) butterflies were observed perching on racemes and flying around aloes. Moths, likely Hawkmoths (*Agrius* sp.), began visiting aloes from 18h17, and visited throughout the night until sunrise (~04:00). Moths comprised most of the floral visits during night-time hours, from 18h17 to 04h00 (n = 93 records; ‘open’ racemes). Other night-time visits by insects were made by grasshoppers and beetles. Common Duiker (*Sylvicapra grimmia*) and Vervet Monkey (*Chlorocebus pygerythrus*) were observed walking around the aloes, but did not interact with the aloes or their flowers in any way. Additionally, bats (species unknown; n = 5) were recorded flying around the aloes during the early morning hours (03h59 – 04h16).

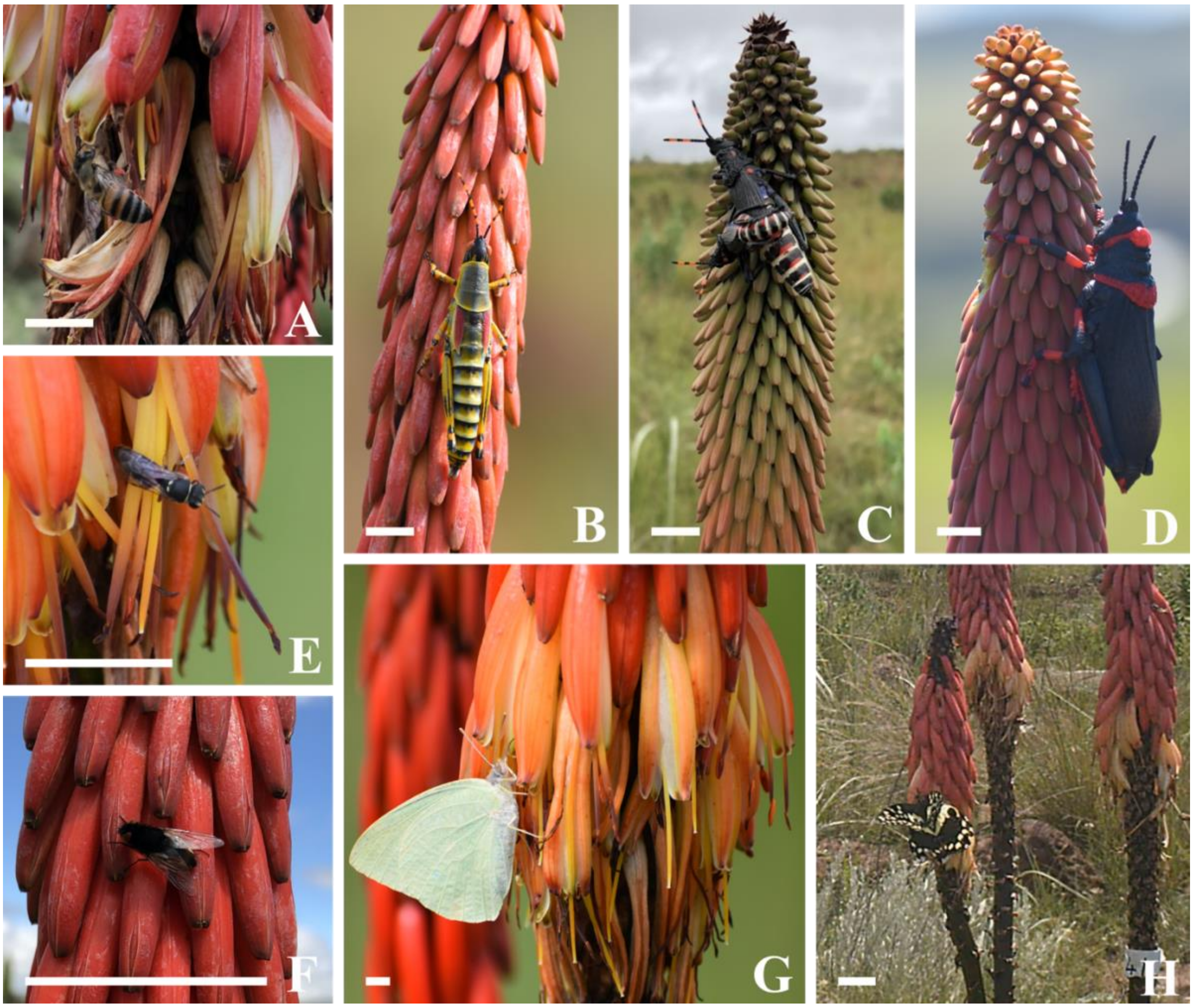

Figure 3.4. Invertebrate floral visitors to *Aloe reitzii* var. *reitzii*: A) African Honeybee (*Apis mellifera*) drinking nectar; B) Elegant Grasshopper (*Zonocerus elegans elegans*) perching on raceme C) Toxic Milkweed Grasshoppers (*Maura rubroornata*) perching; D) Koppie Foam Grasshopper (*Dictyophorous spumans*) perching; E) *Hylaeus* sp. (Hymenoptera) crawling on filaments; F) *Sarcophagidae* sp. (Diptera) sitting on *Aloe reitzii* var. *reitzii* flower buds; G) African Migrant (*Catopsilia florella*) on flowers; H) Citrus Swallowtail (*Papilio demodocus*). Scale bars = 1 cm. Photographs A–G by Jessica Minnaar. Photograph H is a camera trap photo.

Table 3.2. Number of visits of all invertebrate species to *Aloe reitzii* var. *reitzii* plants recorded by camera traps on seven aloes or by researcher observations during the February to March 2020 flowering season. The activity of each invertebrate species was recorded as "Crawling on flowers", "Sitting on flowers", "Nestled in flowers", "Feeding", and "Perching". Where possible, the number of visits to *A. reitzii* flowers are reported.

Order	**Family**	**Species**	**Number of visits**	**Activity**	**Researcher or camera observations**
Coleoptera	Coccinellidae	Ladybug *Harmonia* sp.	-	Crawling on flowers	Researcher
	Melyridae	Spotted Maize Beetle *Astylus atromaculatus*	-	Crawling on flowers	Researcher
	Scarabaeidae	Savanna Fruit Chafer *Dischista rufa*	-	Nestled in flowers	Researcher
Diptera	Calliphoridae	*Chrysoma chloropyga*	-	Sitting on flowers	Researcher
		Chrysoma anthropophaga	-	Sitting on flowers	Researcher
	Drosophilidae	One species	-	Crawling into flowers	Both
	Miltogramminae	*Sarcophagidae* sp.	-	Sitting on flowers	Researcher
	Muscidae	*Antherigona* sp.	-	Sitting on flowers	Researcher
	Syrphidae	*Eumerus* sp.	-	Sitting on flowers	Researcher
Total visitis by Diptera			268		
Hemiptera	Pentominae	One species	-	Crawling on flowers	Researcher
	Chrysomelidae	*Aspidomorpha* sp.	-		Researcher
Hymenoptera	Apidae	African honeybee *Apis mellifera*	3375	Feeding	Both
	Chrysididae	One species	-	-	Researcher
	Halictinae	*Thrinchostoma* sp.	-	Crawling into flowers	Researcher
	Halictidae	*Lassioglossum* sp	-	Crawling into flowers	Researcher
	Colletidae	*Hylaeus* sp.	-	Feeding	Researcher
	Eurytomidae	*Eurytoma aloineae*	-	Crawling into flowers	Researcher
	Vespidae	*Polistes fastidiotus*	-	-	Researcher

Order	Family	Species	Number of visits	Activity	Researcher or camera observations
	Formicidae	-	-	Crawling in and around flowers	Researcher
	Unknown	3 species	-	Crawling in and around flowers	Researcher
Lepidoptera	Nymphalidae	African Monarch *Danaus chrysippus*	1	Perching	Camera
	Papilionidae	Citrus Swallowtail *Papilio demodocus*	2	Perching	Camera
	Pieridae	African migrant *Catopsilia florella*	13	Flying around aloe	Both
		Broad-Boarded Grass Yellow *Eurema brigitta*	5	Perching	Camera
	Sphingidae	*Hawkmoth* sp.	93	Feeding	Camera
Orthoptera	Pneumoridae	Elegant Grasshopper *Zonocerus elegans elegans*	37	Sitting on flowers	Both
		Koppie Foam Grasshopper *Dictyophorous spumans*	2	Sitting on flowers/eating flower buds	Both
	Pyrgomorphidae	Toxic Milkweed Grasshopper *Maura rubroornata*	11	Sitting on flowers	Both
Thysanoptera	Phlaeothripidae	One species	-		Researcher

Pollen load

The pollen load of the various visitors to *A. reitzii* was compared among visitors (both birds and insects) in order to determine whether visitors are pollinators of *A. reitzii* or just floral visitors. *Aloe reitzii* pollen was distinguished from pollen of other flowering plants, such as *Leonotis leonorus*, by its larger size and irregular oval shape (Figure 3.5A). Compared to *Aloe reitzii, L. leonorus* pollen was characterized by its smaller size and circular shape and a possible colpus (Figure 4B). Pollen from *A. reitzii* was the predominant pollen found deposited on both Cape Weavers and Malachite Sunbirds, as well as on bees. *Leonotis leonorus* pollen was only found on Malachite Sunbirds, but was present in smaller quantities compared to *A. reitzii* pollen. *Aloe reitzii* pollen was clearly visible to the naked eye on both male and female Cape Weavers (Figure 3.6A), but only on female Malachite Sunbirds visiting the aloes (Figure 3.6B).

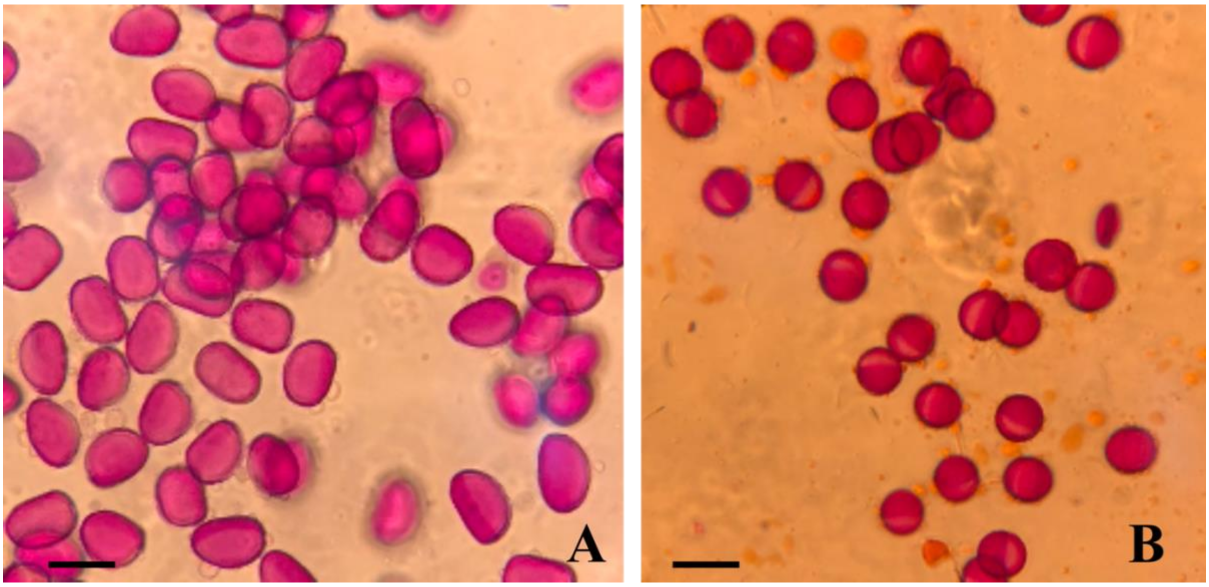

Figure 3.5. A) *Aloe reitzii* var. *reitzii* pollen, characterized by its irregular oval to oblong shape (Scale bar: 250 µm) and B) *Leonotis leonorus* pollen, characterized by its circular shape, with light area possibly a colpus (Scale bar = 200 µm).

A total of 10 Malachite Sunbirds, 11 Cape Weavers and one Greater Double-Collared Sunbird were swabbed for pollen. Both Cape Weavers and Malachite Sunbirds were carrying high numbers of *A. reitzii* pollen (Figure 3.6), with high total pollen load (sum of pollen load from all deposition sites on individual birds) observed: 2208.7 ± 1408.8 pollen grains and 1409.1 ± 732.3 pollen grains, respectively. The Greater Double-Collared Sunbird had a much lower total pollen load (n = 542 pollen grains). Total pollen load was not affected by sex within Cape Weavers and Malachite Sunbirds (β = -971.8 ± 757.1, *P* = 0.2), and did not differ significantly between these bird species (t = 0.83, df = 19, *P* = 0.42; Figure 3.7A).

The main site of *A. reitzii* pollen deposition for both Cape Weavers and Malachite Sunbirds was the throat ($\beta = 1505.4 \pm 767$, $P < 0.05$; Figure 3.6A; Figure 3.6B), with high numbers of pollen observed on the throat of each species (1249 ± 528.7 pollen grains and 1593.4 ± 1434.5 pollen grains, respectively; Figure 3.7B). Although the throat was the main site of pollen deposition for both Cape Weavers and Malachite Sunbirds, pollen load among the main pollen deposition sites did not differ significantly among Cape Weaver ($F = 3$, df = 3, $P < 0.05$) and among Malachite Sunbird ($F = 3.07$, df = 3, $P < 0.05$) individuals. A significant difference was only observed between the throat and the foot among Cape Weaver ($P< 0.05$) and Malachite Sunbird individuals ($P < 0.05$). The lack of statistical significance among pollen deposition sites for Cape Weavers and Malachite Sunbird individuals could be due to the high degree of dispersion of data around the mean, as shown by the coefficient of variation (Table 3.3).

Pollen deposition on the throat did not differ significantly between Cape Weavers and Malachite Sunbirds ($\beta = -574.6 \pm 1051.9$, $P = 0.59$; Figure 3.7B). The crown of each bird species also had high *A. reitzii* pollen deposition, with no significant difference observed between species ($\beta = -402.2 \pm 1051.9$, $P = 0.71$; Figure 3.7B). Additionally, the crown was the main site of pollen deposition for the Greater Double-Collared Sunbird (n = 384 pollen grains). The least amount of pollen was deposited on the bill and foot of each species (Table 3; Figure 3.7B). Similarly, no significant difference in pollen deposition on the bill ($\beta = 662.0 \pm 631.2$, $P = 0.3$) and foot ($\beta = -203.9 \pm 1064.6$, $P = 0.85$) between Cape Weavers and Malachite Sunbirds was observed (Figure 3.7B).

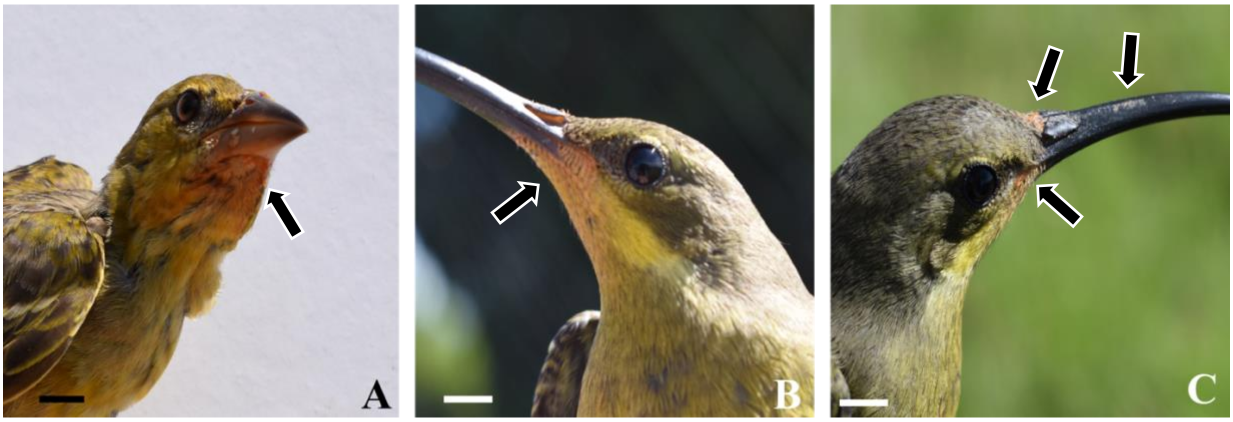

Figure 3.6. Orange pollen of *Aloe reitzii* var. *reitzii*, as shown by the arrows, observed on A) Throat of female Cape Weaver (*Ploceus capensis*); B) Throat of female Malachite Sunbird (*Nectarinia famosa*); C) Forehead, bill and chin of female Malachite Sunbird. Scale = 1cm. Photographs by Jessica Minnaar.

Table 3.3. Pollen load (mean ± SE) of *Aloe reitzii* var. *reitzii* for the main pollen deposition sites for Cape Weavers (*Ploceus capensis*) and Malachite Sunbirds (*Nectarinia famosa*), as shown by bill, crown, foot and throat.

Bird Species	**Pollen deposition site**	**Pollen load (mean ± SE)**	**Coefficient of variation (%)**
Cape Weaver *Ploceus capensis*	Bill	293.5 ± 145.2	117
	Crown	79.45 ± 27.1	164
	Foot	35.3 ± 8.8	140
	Throat	1249 ± 528.7	82
Malachite Sunbird *Nectarinia famosa*	Bill	78.6 ± 29.4	118
	Crown	266.7 ± 121.4	144
	Foot	23.3 ± 10.4	285
	Throat	1593.4 ± 10.4	141

Aloe reitzii pollen was present on all visiting invertebrate guilds, except for thrips, with the highest numbers of *A. reitzii* pollen found on honeybees and solitary bees (2530 ± 1624.4 pollen grains; N = 17; Figure 3.7C). Pollen load differed significantly among the various visiting insect guilds (H = 19.41, df = 6, $P < 0.01$) and among insect species (H = 37.6, df = 21, $P < 0.05$), including between *Apis mellifera* and the grasshopper *Maura rubroornata* ($P < 0.05$), as no pollen was observed on the latter species. Large amounts of *A. reitzii* pollen was observed both honeybees (2302.8 ± 2001.3 pollen grains; n = 12) and *Lasioglossum* sp. (3870 ± 3811.9 pollen grains; n = 4). No pollen was found on the single specimen of Halictinae sp. collected so it was excluded from pollen load analysis. *Aloe reitzii* pollen was mainly deposited on the head and thorax of the bee species. Pollen load did not differ significantly between honeybees and *Lassioglossum* sp. (t = –0.217, df = 14, *P* = 0.831; Figure 3.7A). Although both bird species had higher total pollen loads compared to bees, it did not differ significantly between birds and bees (F = 2.8, df = 3, *P* = 0.06; Figure 3.7A).

Pollinator effectiveness of birds and bees

The relative contributions of birds and insects to the pollination of *A. reitzii* were determined by comparing their effects on the reproductive output (fruit set, seed set and total seed production) of *A. reitzii.* Higher fruit set was observed in the open treatments (45.36 ± 6.8%) than in the bird exclusion treatments (25.75 ± 6.2 %), with the lowest fruit set observed in total exclusion experiments (7.85 ± 2.9%; Figure 3.8A). An overall significant difference in fruit set was observed among the pollinator exclusion experiments and the open treatments (*H* = 22.515, df = 2, *P* < 0.01), but significantly more fruits were produced only by open treatments compared to the total exclusion treatments (*P* < 0.05), with no significant difference between open and bird exclusion treatments (*P* > 0.05), and between bird exclusion and total exclusion treatments (*P* > 0.05).

In addition to the higher fruit set, more seeds per fruit were recorded in open treatments (31.02 ± 2.58 seeds per fruit) compared to bird exclusion (12.38 ± 1.36 seeds per fruit) and total exclusion treatments (5.70 ± 1.36 seeds per fruit). This difference in seed set (proportion of seeds per fruit) among the treatments was significant (*H* = 47.73, df = 2, *P* < 0.0001; Figure 3.8B), as open treatments produced significantly more seeds compared to both bird and total exclusion treatments (*P* < 0.05).

Similar to fruit set and seed set, total seed production also differed significantly among the three treatments (*H* = 36.725, df = 2, *P* < 0.001; Figure 3.8C). Open treatments (3070.28 ± 508.43 seeds) had significantly greater total seed production compared to bird exclusion (826.34 ± 184.91 seeds) and total exclusion treatments (190.38 ± 54.71; *P* < 0.05; Figure 3.8C). Total seed production in bird exclusion treatments was significantly different from the total exclusion treatments (*P* < 0.05; Figure 3.8C).

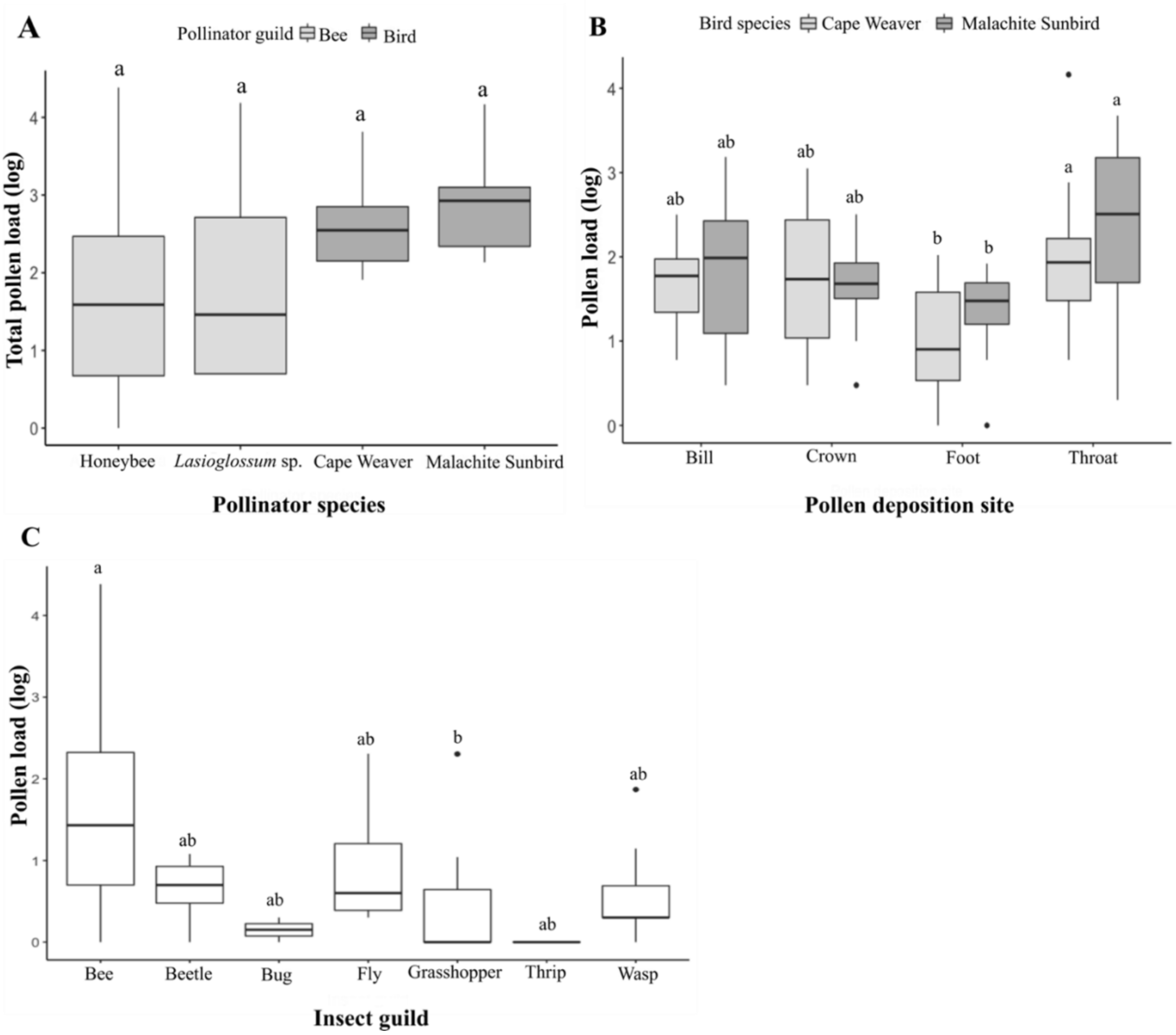

Figure 3.7. Pollen load of *Aloe reitzii* var. *reitzii* pollen on A) Bee (*Apis mellifera* and *Lasioglossum* sp.) and B) bird pollinator guilds (Cape Weaver *Ploceus capensis* and Malachite Sunbird *Nectarinia famosa*); B) main sites of pollen deposition (bill, crown, foot and throat) for Cape Weaver (n = 11) and Malachite Sunbirds (n = 10); and C) insect pollinator guilds: Bees (Order Hymenoptera; n = 17); Beetles (Order Coleoptera; n = 7); Bugs (Order Hemiptera; n = 2); Flies (Order Diptera; n = 7); Grasshoppers (Order Orthoptera; n = 14); Thrips (Order Thysanoptera; n = 2); Wasps (Order Hymenoptera; n = 15). Boxes represent median and inter-quartile range, bars represent minimum and maximum, and solid circles represent outliers. Boxes with different letters are significantly different (Kruskal-Wallis Multiple Comparison Test; $P < 0.05$).

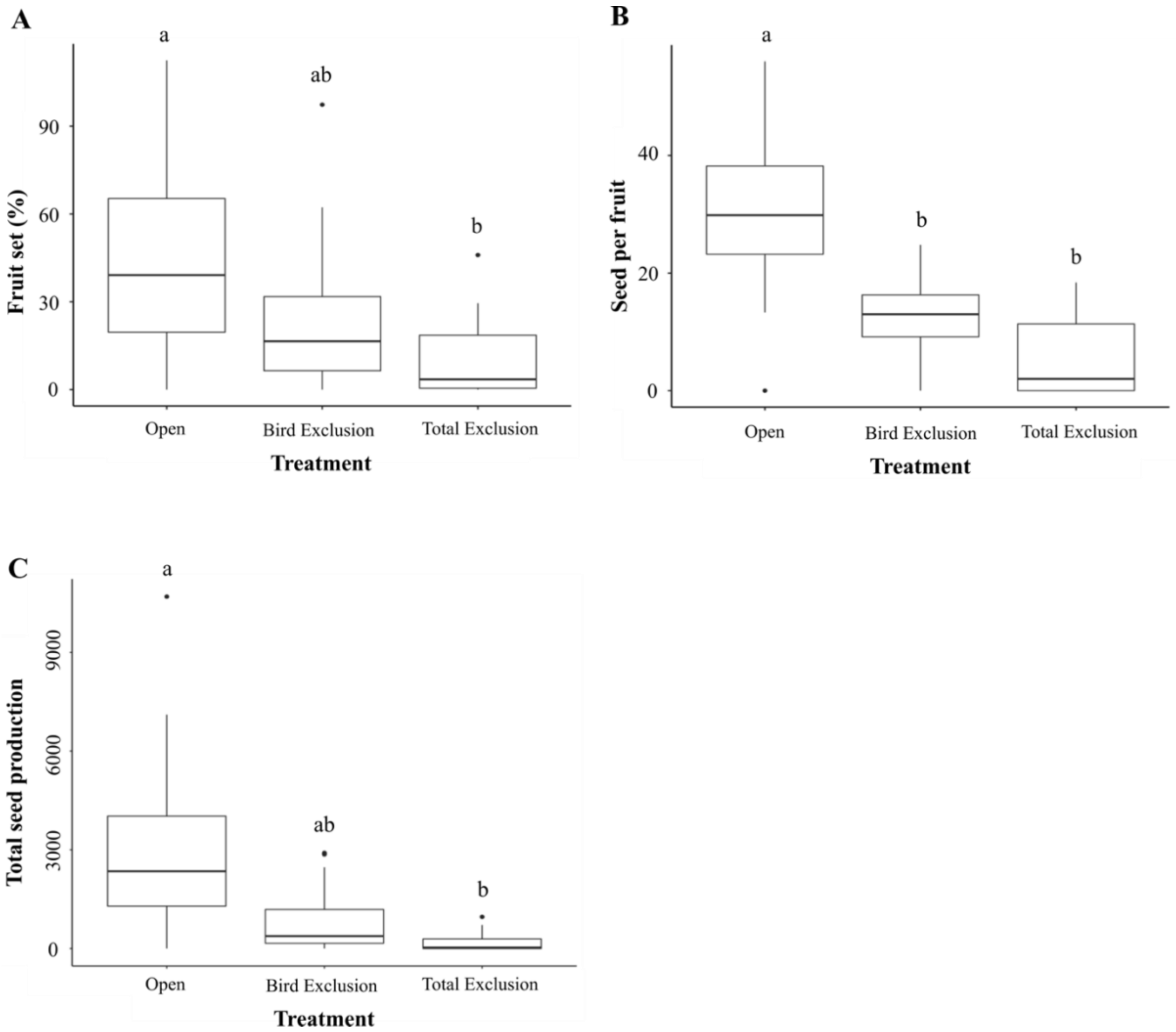

Figure 3.8. Reproductive success of *Aloe reitzii* var. *reitzii* for pollinator selective exclusion experiments during February to March 2020 flowering season (open, bird exclusion and total exclusion treatments), as measured by: A) fruit set (proportion of flowers that set fruit), B) seed set (number of seeds/fruit), C) Total seed production/plant (n = 24 plants/treatment). Boxes represent median and inter-quartile range, bars represent minimum and maximum, and solid circles represent outliers. Treatments with different letters are significantly different (Kruskal-Wallis Multiple Comparison, $P < 0.05$).

Bird pollination system

Nectar properties

Standing crop (unscreened) and nectar availability (screened) nectar volume and concentration were measured to determine whether *A. reitzii* has nectar suggestive of a generalist or specialist bird pollination syndrome. Nectar is continually produced throughout the day, i.e., during daylight hours (Figure 3.9A). Nectar volumes for screened and unscreened racemes were equally affected by time ($\beta = 33.57 \pm 209.62$, $P = 0.16$), with significant changes over time ($\beta = -140.533 \pm 147.081$, $P < 0.01$). Screened nectar volumes significantly decreased over time and standing crop (unscreened) fluctuated over time. Nectar volumes were significantly lower in unscreened plants compared to screened plants (β = -98.32 ± 157.30, *P* < 0.01). Mean screened nectar volumes were lowest at 07:00 (88.54 ± 14.91 μL) and peaked at 17:00 (139.55 ± 38.58 μL). Mean unscreened nectar volumes peaked at 09:00 115.96 ± 21.73 μL) and were lowest at 17h00 (62.73 ± 8.08 μL).

Nectar concentration in screened and unscreened treatments was equally affected by time (β = 0.02 ± 0.08, *P* = 0.83; Figure 3.9B), but in contrast to nectar volume, there were no significant changes over time (β = –0.02 ± 0.05, *P* = 0.66). Nectar concentration did not differ between screened and unscreened treatments (β = –0.08 ± 0.06, *P* = 0.18). Mean nectar concentrations for both screened and unscreened treatments were lowest at 07:00 (screened: 12.0 ± 0.34 % w/w; unscreened: 10.25 ± 0.68 % w/w). However, screened nectar concentration peaked at 11:00 (12.0 ± 0.34 % w/w), whereas unscreened nectar concentration peaked at 13:00 (11.17 ± 0.5 % w/w).

Nectar volume was not correlated with temperature for either screened or unscreened treatments (Screened: Rs = –0.01, *P* = 0.74; Unscreened: Rs = –0.01, *P* = 0.78). Similarly, screened and unscreened nectar concentrations were not correlated with temperature (Screened: R_S = –0.001, *P* = 0.52; Unscreened Rs = 0.012, *P* = 0.19). Similar to temperature, humidity was also not correlated with nectar volume for screened and unscreened treatments (Screened: Rs = –0.01, *P* = 0.652; Unscreened: Rs = –0.01, *P* = 0.61), nor for nectar concentration (Screened: Rs = –0.002, *P* = 0.36; Unscreened: Rs = 0.008, *P* = 0.31).

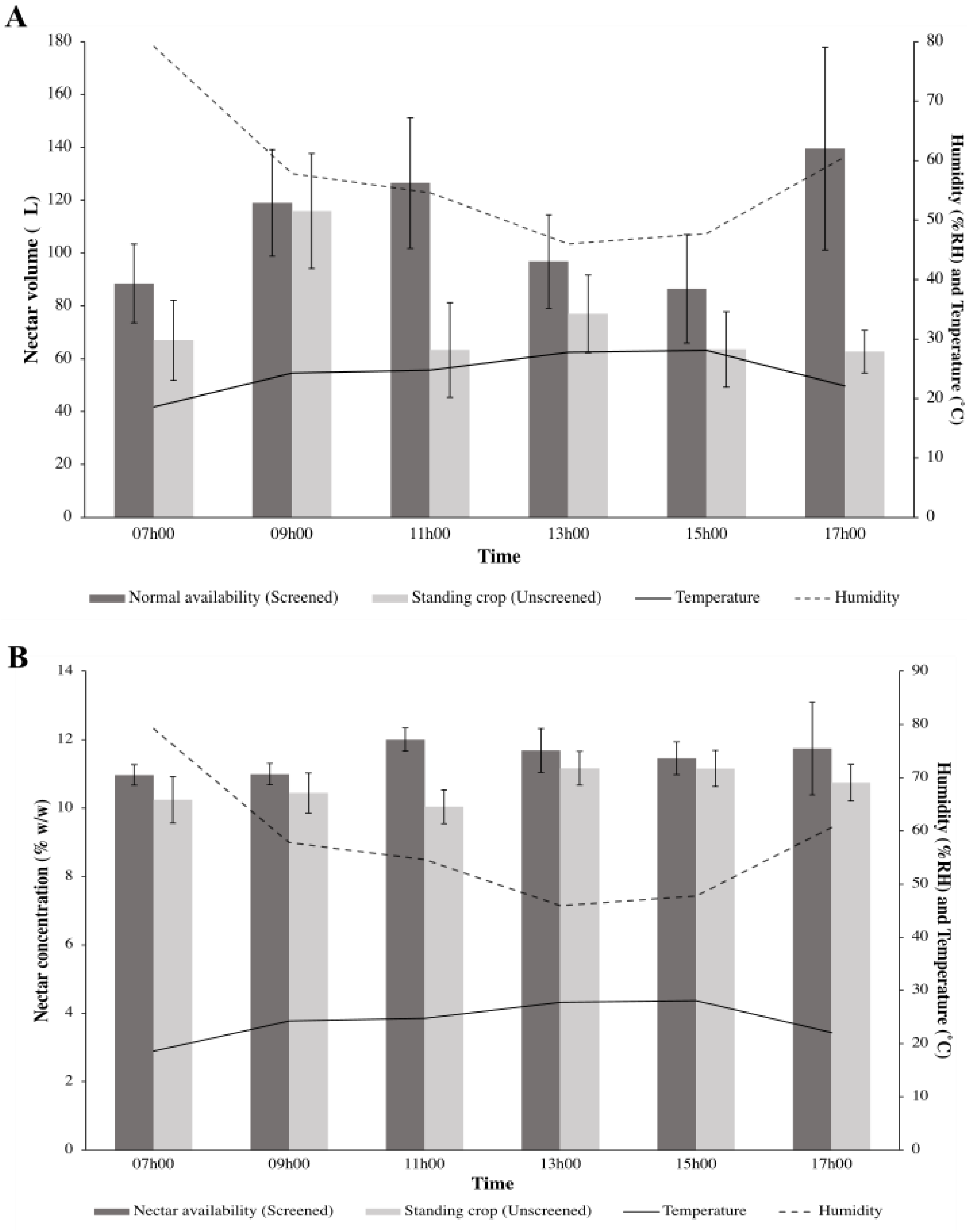

Figure 3.9. A) Nectar volume (μL; mean ± SE) and B) Nectar concentrations (% w/w; mean ± SE) for screened (dark grey) and unscreened (light grey) treatments of *Aloe reitzii* var. *reitzii* during the February to March 2020 flowering season (n ≤ 10 flowers per treatment per time period; *N* = 11 racemes per treatment), measured at two-hour intervals from 07h00 to the last two hour interval at 17h00 (daylight hours). Mean temperature (°C) shown by solid grey line and mean humidity (% RH) shown by dashed grey line.

Bill morphology vs. floral morphology

The length and curvature of the *Aloe reitzii* flowers were compared with that of the main bird species visiting the flowers to infer which bird species could optimally come into contact with the reproductive organs of *A. reitzii,* thus effectively pollinating its flowers. *Aloe reitzii* flowers are characterized by long, narrow perianth tubes (44.96 ± 0.45 mm), with strongly exserted stamens (57.58 ± 0.83 mm) and styles (55.34 ± 0.78 mm; Figure 3.10E). Cape Weavers have significantly shorter bills (21.85 ± 0.0002 mm; $N = 7378$) compared to Malachite Sunbirds (32.34 ± 0.001 mm*;* $N = 1953$; $\beta = 9.09 \pm 0.13$, $P < 0.001$; Figure 3.10; Table 3.4). Bill length of both bird species was significantly shorter than the perianth length of *A. reitzii* flowers ($P < 0.001$). Bill lengths of Cape Weavers and Malachite Sunbirds were equally affected by sex, with males having longer bills compared to females ($\beta = 1.12 \pm 0.08$, P < 0.001). Male Malachite Sunbirds had significantly longer bills compared to male Cape Weavers ($\beta = 1.89 \pm 0.16$, $P < 0.001$; Figure 3.10A; Figure 3.10C). However, the bill length of both the Cape Weavers and the Malachite Sunbirds were weakly correlated with perianth tube length (Figure 3.11A), stamen length (Figure 3.11B) and style length (Figure 3.11C) of *A. reitzii* (Table 3.5).

Malachite Sunbirds have a significantly more curved bill compared to Cape Weavers ($\beta = 174.53 \pm 2.05$, $P < 0.001$; $N = 11$; Table 3.4*).* Bill curvature was not affected by sex for Cape Weavers or Malachite Sunbirds ($\beta = 1.84 \pm 3.037$, $P = 0.55$). Bill curvature of Cape Weavers was not correlated to the perianth tube curvature (Perianth curvature: 175.4 ± 1.31° ; $R_S = 0.14$, $P = 0.26$; Figure 3.11D), and it differed significantly ($U = 277$, $P < 0.001$). There was also no correlation in the bill curvature of Malachite Sunbirds with perianth tube curvature of *A. reitzii* ($R_S = 0.24$, $P = 0.15$; Figure 3.11D), despite the fact that they did not differ ($U = 155$, $P = 0.89$).

Table 3.4. Bill morphology measurements (mean ± standard error) of Cape Weaver (*Ploceus capensis*) and Malachite Sunbird (*Nectarinia famosa*) as measured by bill length (mm) and bill curvature (°). The number of individuals (*N*) used for each measurement is also shown.

Bird species	**Bill length (mm; Mean + SE)**	***N***	**Bill curvature (˚; Mean ± SE)**	***N***
Cape Weaver	21.85 ± 0.0002	7378	175.4 ± 1.31	11
Malachite Sunbird	32.34 ± 0.001	1953	170.5 ± 0.001	10

Table 3.5. Simple linear regression results comparing bill length of Cape Weavers (*Ploceus capensis*) and Malachite Sunbirds (*Nectarinia famosa*) to selected floral features of *Aloe reitzii* var. *reitzii* (Rs, top and *P*-value, bottom), namely perianth tube length (mm), stamen length (mm) and style length (mm). Mean and standard error (SE) for corolla tube length (mm), stamen length (mm) and style length (mm) are also presented.

Floral morphology (mm)	**Mean ± SE (mm)**	**R_S and *P* - value**	
		Cape Weaver	**Malachite Sunbird**
Perianth tube length	44.96 ± 0.45	0.01 $P < 0.001$	0.04 $P < 0.001$
Stamen length	57.58 ± 0.83	0.03 $P < 0.001$	0.003 $P < 0.001$
Style length	55.34 ± 0.78	0.01 $P < 0.001$	0.04 $P < 0.001$

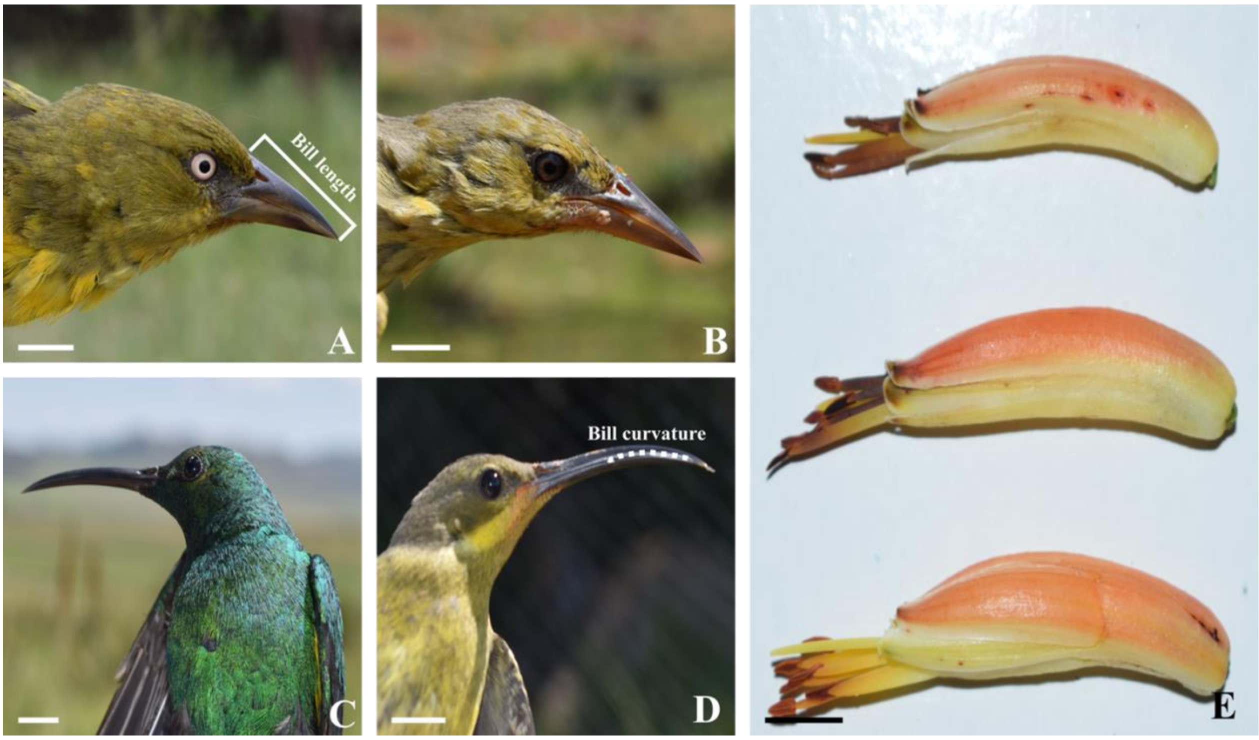

Figure 3.10. Bill comparisons of A) Male Cape Weaver (*Ploceus capensis*); Female Cape Weaver; C) Male Malachite Sunbird (*Nectarinia famosa*); D) Female Malachite Sunbird; and E) flowers of *Aloe reitzii* var. *reitzii* showing the strongly exserted anthers and style with stigma at its tip. Bill length was measured from the union of the bill with the base of the skull to the tip of the bill (A) and bill curvature of the tip of the bill was measured (D). Scale = 1 cm. Photographs by Jessica Minnaar.

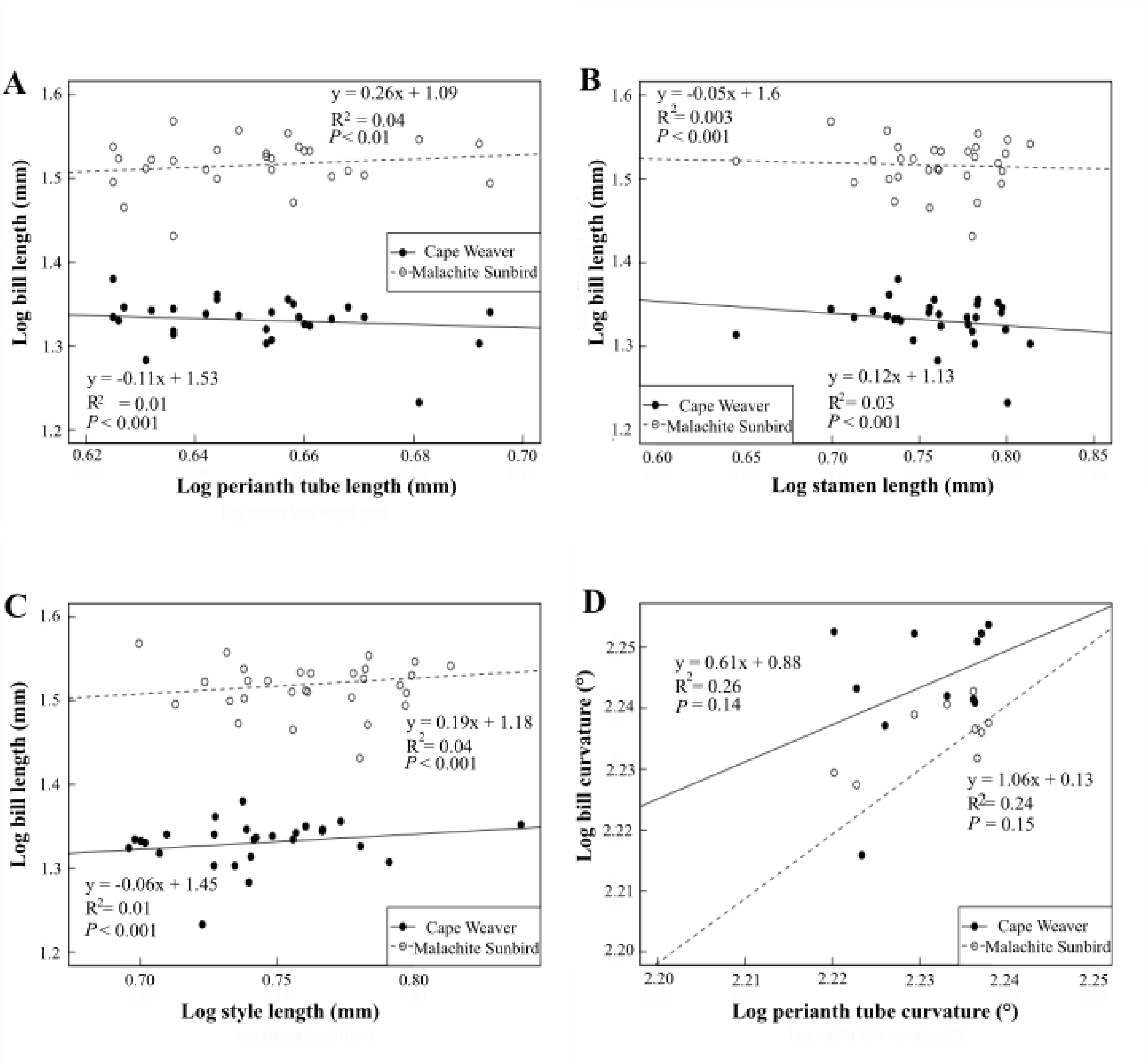

Figure 3.11. Comparison of the bill length (mm) of Cape Weaver (*Ploceus capensis*; n= 7378), and Malachite Sunbird (*Nectarinia famosa*; n = 1953) with A) Corolla tube length (n=30), B) Stamen length (mm; n = 30), C) Style length (mm; n = 27) of *Aloe reitzii* var. *reitzii.* D) Comparison of bill curvature of Cape Weaver (n =11) and Malachite Sunbird (n = 10) with the perianth tube curvature (n = 30) of *Aloe reitzii* var. *reitzii.* Cape Weaver is represented by black circles, and correlation by solid line. Malachite Sunbird is represented by open circles, and correlation represented by dotted line.

DISCUSSION

Like most ornithophilous *Aloe* species, *A. reitzii* is predominantly pollinated by birds, which is consistent with its "ornithophilous" floral traits. Short-billed and long-billed specialist nectarivores are both effective pollinators of *A. reitzii*, as shown by the abundance of Cape Weavers and Malachite Sunbirds at *A. reitzii* flowers, and the significantly high amounts of *A. reitzii* pollen found on both species. This result is supported by the floral traits and nectar properties of *A. reitzii* being intermediate between a generalist and specialist bird pollination syndrome (Botes *et al.*, 2008; Johnson and Nicolson, 2008). Cape Weavers and Malachite Sunbirds are therefore co-pollinators of *A. reitzii,* but due to their greater abundance and more frequent visitation *A. reitzii* flowers, Cape Weavers are the primary pollinators of *A. reitzii*. Although honeybees (*Apis mellifera*) were highly abundant visitors, they probably play only a minor role in the pollination of *A. reitzii*, as seen by the low seed set in bird exclusion treatments.

Pollinator effectiveness of birds and bees

Cape Weavers comprised majority (57.5%) of overall bird visits, and although Malachite Sunbirds comprised most specialist bird visits, they only contributed 33.3% of overall bird visits. These results are consistent with findings of Symes (2017) during the 2014 flowering period, when Cape Weavers contributed most (~60%) visits, while Malachite Sunbirds only contributed 30% of bird visits. Although Malachite Sunbirds comprised most visits during the 2017 flowering period (49%), Cape Weavers were still abundant visitors to *A. reitzii* flowers (41%; Payne, 2019). Therefore, Cape Weavers have consistently been abundant at *A. reitzii* during multiple flowering periods, whereas the abundance of Malachite Sunbirds at this population of *A. reitzii* has fluctuated each study year.

The fluctuation in abundance of Malachite Sunbirds at this study population of *A. reitzii* over the past few years resulted in their contributing only 27.1% of overall bird visits in 2014 (Symes, 2017), 49% in 2017 (Payne, 2019), and 33% in 2020. Both specialist and generalist nectarivores are known to increase in abundance in response to increasing food availability, especially during the winter months when food is scarce (Symes *et al.*, 2008; Forbes *et al.*, 2009; Kuiper *et al.*, 2015). Of these bird species, Cape Weavers and Malachite Sunbirds increase in abundance in response to the flowering of winter-flowering aloes, such as *A. marlothii* (Symes *et al.*, 2008) and *A. ferox* (Forbes *et al.*, 2009; Kuiper *et al.*, 2015). Malachite Sunbirds are known to seasonally track flowering plants based on their nectar supply (Skead, 1967; Craig and Hulley, 1994; Symes *et al.*, 2001), resulting in their fluctuating abundances in successive years (Skead, 1967), while an increase in Cape Weaver abundance at aloes can be attributed to the fact that they are “addicted probers” of nectar at *Aloe* flowers (Oatley, 1964).

Nectar-feeding birds, such as hummingbirds, display trap lining behaviour in response to nectar rewards and nectar availability of flowering plants (Temeles *et al.*, 2006). Territorial behaviour is also common, especially in nectar-rich areas, wheremale nectar-feeding birds hold territories not only to meet their energy requirements, but also to attract females as potential mates (e.g., purple-throated carib hummingbirds, *Eulampis jugularis*; Temeles and Kress, 2010). Malachite Sunbirds are known to follow an annual "tourist" pattern (Craig and Hulley, 1994; Symes *et al.*, 2001), increasing in abundance in response to the flowering of *Leonotis leonorus* (L.) R. Br. in spring, *Protea caffra* in autumn (Symes *et al*., 2001), and *Aloe* spp. in winter (e.g., *A. marlothii*, Symes *et al*., 2008; and *A. ferox*, Forbes *et al*., 2009), often arriving in flocks (Forbes *et al.*, 2009; Kuiper *et al.*, 2015). Additionally, Malachite Sunbirds have been shown to act as "shoppers" within an annual "tourist" activity pattern at *L. leonorus*, increasing in abundance in response to the copious nectar production during their flowering period (Craig and Hulley, 1994; Symes *et al.*, 2001). Since Malachite Sunbirds are consistent visitors at this population of *A. reitzii* each year, their fluctuating abundance at *A. reitzii* suggests they may be "shoppers" at *A. reitzii* during the summer months, within their "tourist" activity pattern. The species richness of birds visiting *A. reitzii* and the percentage of flowering plants in successive years should be investigated to infer if the fluctuating abundance of Malachite Sunbirds at this population of *A. reitzii* occurs in response to changing food/nectar availability in this region. However, it should be noted that the abundance of Malachite Sunbirds was higher in 2014 when more aloes were recorded flowering (29%; Payne, 2019) compared to 2014 (18.9%; Symes, 2017), suggesting the abundance of Malachite Sunbirds fluctuates in response to changing food availability. Additionally, the number of recaptures of Malachite Sunbird individuals should be used to determine if the same individuals return annually, thereby not opportunistically feeding on aloes ("shoppers"), confirming the "tourist" activity pattern of Malachite Sunbird at *Aloe*.

Aloe reitzii pollen was present on Cape Weavers, Malachite Sunbirds and Greater Double-Collared Sunbirds (*Cinnyris afer*), all of which fed on *A. reitzii* nectar. Although more *A. reitzii* pollen was present on Cape Weavers, comparable pollen loads were also observed on Malachite Sunbirds, indicating both bird species are effective pollinators of *A. reitzii.* Despite the fact that *A. reitzii* pollen, albeit in low quantities, was present on Greater Double-Collared Sunbirds, their low abundance and infrequent visitation at *A. reitzii* suggests they do not significantly contribute to the pollination of *A. reitzii.* In 2014, Greater Double-Collared Sunbirds contributed 12.5% of bird visits (Symes, 2017), while only contributing only 3.9% of visits in 2017 (Payne, 2019). The abundance of Greater Double-Collared Sunbirds at *A. reitzii* flowers in 2020 appeared to be low as they were not recorded feeding on aloes by camera traps and were only observed feeding on two aloes (J. Minnaar, *pers. obs.*). Although not highly abundant, Amethyst Sunbirds (*Chalcomitra amethystina*) were frequent visitors, however their contribution to the pollination of *A. reitzii* could not be determined as they were not swabbed for pollen. The low abundance of other specialist nectarivores visiting *A. reitzii* can be attributed to the territorial behaviour of Malachite Sunbirds. Malachite Sunbirds are known to be highly territorial (Hargreaves *et al.*, 2004; Geerts and Pauw, 2009), especially at dense populations of plants with long-tubed nectar-producing flowers (Skead, 1967), reducing the abundance of shorter-billed sunbirds (Geerts and Pauw, 2009). Therefore, the high pollen loads of Cape Weavers and Malachite Sunbirds, coupled with their abundance at *A. reitzii* flowers indicate they are both important, effective pollinators of *A. reitzii*.

Similar to previous studies of *A. reitzii* (Symes, 2017; Payne, 2019), honeybees (*Apis mellifera*) were highly abundant at *A. reitzii* flowers, contributing the majority of insect visits. The high abundance of honeybees in the study area likely cannot be attributed to the presence of beehives on the farm, as the landowner did not mention the presence of beehives in the area (S. Payne *pers. comm.*). Additionally, moths were abundant visitors during the night-time hours; consistent with previous findings (Payne, 2019). However, their role in the pollination of *A. reitzii* is yet to be determined by catching them and measuring pollen loads. Additionally, night-time exclusion experiments could be conducted to determine their contribution to the reproductive output of *A. reitzii*. Although honeybees and solitary bees (*Lasioglossum* sp.) had similar *A. reitzii* pollen loads comparable to that of bird pollinators, they did not greatly contribute to the reproductive output of *A. reitzii*, as bird-excluded plants had significantly lower seed set compared to plants that included all pollinators. This finding cannot be explained by autogamy as *A. reitzii* requires pollen transfer between flowers for successful seed set (Chapter 2).

Bees are shown to play a negligible or minor role in the pollination of many ornithophilous *Aloe* species (e.g., *A. ferox*, Stokes and Yeaton, 1995, Botes *et al.*, 2009a; *A. speciosa* and *A. arborescens*, Botes *et al.*, 2009a), particularly those with long-floral tubes and strongly exserted filaments (Botes *et al.*, 2009a). Despite the abundance of bees, aloes with dense racemes of tubular flowers set almost no seeds where birds were excluded (e.g., *A. ferox* and *A. marlothii*, Hargreaves *et al.*, 2012), as the strongly exserted anthers prevent larger bee species, such as honeybees, from entering their floral tubes and effectively transferring pollen onto the stigmas (Stokes and Yeaton, 1995; Botes *et al.*, 2009a; Symes *et al.*, 2009). Nonetheless, although *A. reitzii* has dense racemes of long tubular flowers (Figure 3.1A), treatments where birds were excluded still set fruit and seeds, suggesting bees do effectively transfer pollen onto the stigmas of *A. reitzii.* Compared to *A. ferox* and *A. marlothii*, the style protrudes and presents the stigma in *A. reitzii* (Figure 3.10E), indicating that bees are able to successfully pollinate its flowers without having to enter the floral tubes. Therefore, bees play a minor role in the pollination of *A. reitzii.* However, the reduced fecundity observed in bird-excluded treatments suggests that bees are likely transfering self-pollen, reducing seed set arising from geitonogamous selfing.

Since *A. reitzii* is self-compatible it is highly susceptible to geitonogamous selfing, which results in inbreeding depression and pollen discounting (Harder and Barrett, 1996; Richards, 1997; Barrett, 2002, 2003; Chapter 2). Bird-excluded treatments produced similar fruit set to plants that included birds (open treatment), but significantly lower seed set was observed in bird-excluded plants, suggesting geitonogamous selfing by bees resulted in reduced fecundity. Poor pollen quantity and quality can result in reduced seed set, especially in hermaphroditic plants with late-acting self-incompatibility mechanisms that rely on outcrossing for seed set (Aizen and Harder, 2007), such as those commonly present in many *Aloe* species (Appendix A). Insects are known to carry and deposit low-quality pollen (self-pollen that results in reduced fecundity in self-incompatible aloes; Hargreaves *et al.*, 2012), suggesting the low reproductive output observed in bird-excluded *A. reitzii* plants may also be a result of self-pollen deposition by bees.

Pollen-collecting bees are poor-pollinators of aloes, as they prefer visiting male-phase flowers during fresh anthesis, when the stigmas are still immature and shorter than the anthers, thus not making contact with stigmas (Botes *et al.*, 2009a), and reducing the reproductive output of plant populations (Hargreaves *et al.*, 2010). In *A. reitzii*, most pollen had been removed once the stamens had elongated (J. Minnaar, *pers. obs*.), indicating the high pollen removal by these highly abundant visitors. Therefore, the high pollen loads observed on both honeybees and solitary bees, and their poor contribution to the reproductive output of *A. reitzii* suggest that they may be pollen thieves (Hargreaves *et al.*, 2009). However, fruit produced in bird-excluded plants did not significantly differ from plants that allowed all floral visitors, suggesting bees are still pollinating the flowers. The significant difference in seed set of bird-excluded plants compared to plants that allowed all floral visitors suggests bees likely facilitate geitonogamous self-pollination (Chapter 2). However, the feeding behaviour of bees at *A. reitzii* flowers and the amount of pollen deposited on stigmas during their visits need to be more fully investigated to determine the extent to which they may be pollen thieves, and the amount of self-pollen deposited on flowers for successful pollination.

Bird pollination syndrome

Aloe reitzii produces large nectar volumes with low sugar concentration, characteristic of a generalist bird pollination system (Johnson and Nicolson, 2008), further supporting Cape Weavers as the primary pollinator of *A. reitzii*. Nectar results shown here contrast with those of Symes (2017), in which nectar properties followed a specialist pollination syndrome in 2014 but are similar to those recorded by Payne (2019). This could be attributed to change in mean annual rainfall in successive years. Environmental parameters are shown to influence nectar secretion and composition, as plants exposed to higher humidity and greater temperatures produce greater volumes of more dilute nectar (Wyatt *et al*., 1992). In 2017, the average temperature when nectar measurements were taken was 21.7 °C, compared to the higher temperature (24.25°C) and humidity (57 % w/w) in the 2020 nectar study. Therefore, inconsistent nectar results could be attributed to fluctuating environmental parameters, with greater volumes of more dilute nectar, characteristic of a generalist pollination syndrome (Johnson and Nicolson, 2008) occurring in response to increased temperature and humidity in 2020.

The peak in Malachite Sunbird visitation to *A. reitzii* corresponds to times of day when nectar volume was at its lowest and nectar concentration at its highest, at 12h00 (nectar volume: 63.3± 17.87 µL – 77 ± 14.7 µL; nectar concentration: 10.04 ± 0.49– 11.17 ± 0.5 % w/w) and 17h00 (nectar volume: 62.73 ± 8.08 µL; nectar concentration: 11.17 ± 0.05 % w/w). At these times, nectar properties are most similar to those found in plants pollinated by specialist nectarivores (Johnson and Nicolson, 2008). Therefore, the intermediate floral traits and nectar properties of *A. reitzii* promote effective pollination from both Cape Weavers and Malachite Sunbirds.

Many aloes that are pollinated by both specialist and opportunistic nectarivores have nectar properties consistent with a generalist pollination syndrome, and specialist nectarivores, such as Malachite Sunbirds, have been shown to feed on aloes with generalist nectar properties (Kuiper *et al.*, 2015). Although specialists prefer nectar with high sugar (sucrose) concentration (Johnson and Nicolson, 2008), Malachite Sunbirds have been observed feeding on aloes, such as *A. ferox*, despite its low sugar concentrations (Kuiper *et al.*, 2015). Malachite Sunbirds are capable of adjusting their volumetric intakes at concentrations between 10% and 25% and are therefore capable of feeding on plants with nectar properties of a generalist pollination system, optimizing the hours at which nectar properties most suit their physiological requirements (Brown *et al.*, 2010), as seen by their feeding patterns in *A. reitzii*. Therefore, the copious dilute nectar produced by *A. reitzii* flowers does not deter specialist nectarivores from feeding on flowers (also seen in *A. tenuior* Haw.; Johnson *et al.*, 2006), and subsequently cross-pollinating flowers as they feed from plant to plant.

Floral traits and nectar properties are commonly used to infer primary bird pollinators in *Aloe* species with an ornithophilous pollination syndrome. Short-billed generalists are primary pollinators of aloes with short-tubed flowers that produce large volumes of relatively dilute nectar ("generalist pollination syndrome", Botes *et al.*, 2008; Johnson and Nicolson, 2008; Symes and Nicolson, 2008; Symes *et al.*, 2009; Hargreaves *et al.*, 2012). The opposite is true for long-billed specialist nectarivores, which are pollinators of long-tubed aloes with small volumes of relatively dilute nectar ("specialist pollination syndrome"; Botes *et al.*, 2008; Johnson and Nicolson, 2008; Hargreaves *et al.*, 2012). Cape Weavers are frequent visitors to many "generalist" aloes, often playing a primary role in their pollination, such as *A. ferox* (Botes *et al.*, 2008; Forbes *et al.*, 2009; Hargreaves *et al.*, 2012; Kuiper *et al.*, 2015) and *A. marlothii* (Symes and Nicolson, 2008), while Malachite Sunbirds are pollinators of "specialist" *Aloe* species (*A. pluridens* Haw. and *A. lineata* (Aiton) Haw. var. *muirii* Marloth; Botes *et al.*, 2008).

Contrary to predictions, Malachite Sunbirds are also effective pollinators of the short-tubed *A. kraussii* Baker, which has a bimodal pollination system (Hargreaves *et al.*, 2019). In comparison to *Aloe* species with a "specialist" or "generalist" pollination syndrome, *A. reitzii* has floral traits intermediate between these floral syndromes – as seen by their long perianth tubes and copious dilute nectar (and exserted anthers and stigmas) – accounting for both specialist and generalist nectarivores as effective pollinators, respectively. However, *A. reitzii* does not have a true bimodal pollination system as its floral traits are not intermediate between two different pollination systems where two sets of pollinators from different pollinator guilds are equally effective co-pollinators (Manning and Goldblatt, 2005). An example of a true bimodal pollination system using two different pollinator groups is seen in *A. kraussii* which has floral traits intermediate between those of bird and bee pollination (Hargreaves *et al.*, 2019). In contrast, *Aloe reitzii* has floral traits intermediate between two bird pollinator guilds, specialist and generalist bird pollination, enabling effective pollination by both Cape Weavers and Malachite Sunbirds, indicating a possible bimodal pollination system within a pollinator guild.

Aloe reitzii pollen was mainly deposited on the throat of both Cape Weavers and Malachite Sunbirds, where they come into contact with the reproductive organs of *A. reitzii* flowers, pollinating flowers as they feed on nectar. The throat, crown and bill are main pollen deposition sites for bird visitors to aloes (Botes *et al.*, 2008; Symes and Nicolson, 2008; Diller *et al.*, 2019). However, pollen deposition on the throat of visiting birds seems to be a common pattern observed in aloes with strongly exserted filaments, such as seen here in *A. reitzii*, and also in *A. marlothii* (Symes and Nicolson, 2008), for example. Even though a plant's floral traits are correlated with its primary pollinators (Fenster *et al.*, 2004), short-billed generalists are shown to be effective pollinators of aloes with mid-length to long tubular flowers (e.g., *A. ferox*, Diller *et al.*, 2019). The larger heads and thicker bills of generalist nectarivores enable them to spread apart the exserted anthers and stigmas as the feed on nectar, facilitating effective pollen deposition on their feathers (Diller *et al.*, 2019). Cape Weavers feed on aloe nectar by probing open flowers at the bottom of the raceme, by separating the tepals at the mouth of flowers and pushing their bill into the perianth to feed on copious nectar while perching on the raceme peduncle, or they feed upside down by perching on closed flowers on the raceme (Figure 3.1F; detailed in Botes *et al.*, 2009a). In both feeding positions, Cape Weavers fully insert their heads into *A. reitzii* flowers to feed on nectar (Figure 3.1D), resulting in large quantities of pollen being deposited on their throat and crown as they rub against the filaments. Therefore, key facial regions come into contact with reproductive parts of flowers, enabling cross-pollen transfer in *A. reitzii*, as they move from plant to plant. Hence, floral traits should not solely be taken into consideration when defining primary pollinators, as the feeding behaviour of birds also plays a role in whether they are effective outcrossers.

Malachite Sunbirds are important pollinators of many plants with long tubular flowers (Geerts and Pauw, 2009). In *A. ferox*, the slender bills, small heads and long tongues of specialist nectarivores do not make effective contact with the reproductive parts of flowers, so they act as nectar robbers (Diller *et al.*, 2019). Since the floral tubes of *A. reitzii* are longer than the bill of Malachite Sunbirds, they have to stretch their necks in order to reach the nectar at the base of flowers, often extending their tongues to do so (Geerts and Pauw, 2009). Their feeding is made easier as the curved perianth tubes of *A. reitzii* match the decurved bills of Malachite Sunbirds, and they are able to efficiently feed on nectar without manipulating flowers through their perching position (Johnson *et al.*., 2020). Malachite Sunbirds feed on *A. reitzii* flowers by perching on the peduncle below the inflorescence and feed on basal flowers by reaching their heads upwards (Figure 3.1G); a common pattern in specialist nectarivores feeding on aloes (Botes *et al.*, 2008). Therefore, they are able to come into contact with the strongly exserted filaments of *A. reitzii* as they feed (Figure 3.10E), enabling pollen deposition on their throats and ensuring cross-pollen transfer as they feed from plant to plant. Thus, both Cape Weavers and Malachite Sunbirds are effective pollinators of *A. reitzii*, as the strongly exserted filaments come into contact with their feathers as they feed, enabling successful cross-pollen transfer as they move from plant to plant. Therefore, floral characteristics and bill morphology are not always indicative of the most effective bird pollinators of aloes. This is evident in *A. ferox* (mid-length floral tubes; Hargreaves *et al.*, 2012), where short-billed weavers (*Ploceus* spp.) are highly effective pollinators compared to long-billed specialist nectarivores and honeybees, as they deposit more pollen on a per-visit basis (Diller *et al.*, 2019). However, Cape Weavers and Malachite Sunbirds were also observed feeding on the open, mature, basal flowers between racemes on the same aloe (J. Minnaar, *pers. obs.*). Since aloe flowers mature acropetally on the raceme (Botes *et al.*, 2008), this foraging behaviour may promote geitonogamous selfing and result in inbreeding depression (Harder and Barrett, 1996; Chapter 2).

Conclusion and recommendations for future studies

Aloe reitzii is predominantly pollinated by birds, which is consistent with its ornithophilous floral traits. The long floral tubes and generalist nectar properties of *A. reitzii* enable effective pollination by both generalist and specialist nectarivores. Both Cape Weavers and Malachite Sunbirds are effective pollinators of *A. reitzii*, but due to their greater abundance and more frequent visitation, Cape Weavers are concluded to be the primary pollinators of *A. reitzii*. The fluctuating abundance of Malachite Sunbirds at this population of *A. reitzii*, suggests the sunbirds may be “shoppers”, increasing in abundance in response to nectar availability. However, the relationship between bird species richness and the number of flowering plants in successive years needs to be investigated to determine if Malachite Sunbird abundance at this aloe population is indeed influenced by nectar availability and summer rainfall. Additionally, the abundance of birds at different sub-populations of *A. reitzii* across its range should be investigated.

Since only one Greater Double-Collared Sunbird and no Amethyst Sunbirds were swabbed for pollen, the roles of these specialist nectarivores in the pollination of *A. reitzii* need to be clarified. Although bees are shown here to make a minor contribution to the pollination of *A. reitzii*, the foraging behaviour of bees at the flowers should be investigated to determine the amount of self-pollen deposited on stigmas during floral visits relative to the total pollen load carried by bees. This would support or refute the hypothesis put forward here that the bees are mainly transferring self pollen within a plant, resulting in geitonogamous self-pollination. Moths were confirmed as frequent night-time visitors to *A. reitzii*, as observed by Payne (2019). However, their role in the pollination of *A. reitzii* is yet to be determined by investigating: i) moth species visiting *A. reitzii* flowers and whether they carry any *A. reitzii* pollen, as well as observing their behaviour at *A. reitzii* flowers (perching on flowers, drinking nectar and if they make contact with reproductive organs) and ii) night-time nectar production by, and scent of, *A. reitzii* to determine if nectar is produced during the hours when moths are most frequently visiting *A. reitzii* flowers; iii) night-time exclusion experiments to determine their contribution to the reproductive output of *A. reitzii*.

In previous studies of *A. reitzii*, both birds and insects, particularly bees, were frequent visitors to *A. reitzii* flowers (Symes, 2017; Payne, 2019). However, their role in the pollination of *A. reitzii* was not investigated. This study has shown that *A. reitzii* is primarily pollinated by birds, while bees play only a minor role in the pollination of *A. reitzii*, confirming the relative pollinator effectiveness of bird and bee visitors to *A. reitzii* (Objective 2). Generalist (Cape Weavers) and specialist (Malachite Sunbirds) nectarivores were both frequent visitors to *A. reitzii* in previous studies (Symes, 2017; Payne, 2019) but their abundance at *A. reitzii* flowers was not consistent. Additionally, Symes (2017) recorded nectar properties consistent with a specialist bird pollination syndrome, while nectar properties in Payne (2019) were suggestive of a generalist bird pollination syndrome. Both Cape Weavers and Malachite Sunbirds are important and effective pollinators of *A. reitzii*. However, the abundance of Cape Weavers shown here is similar to previous studies (Symes, 2017; Payne, 2019), indicating Cape Weavers are the primary pollinator of *A. reitzii*. This is supported by the generalist nectar properties shown here, which are similar to nectar properties recorded by Payne (2019). Hence, the objective to infer the relative importance of generalist and specialist nectarivores in the pollination of *A. reitzii* was achieved. Therefore, this study accomplished the aim to compare the role of birds and insects in the pollination of *A. reitzii* and establish the relative importance of generalists versus specialist nectarivores.

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CHAPTER FOUR

Fruit and seed predation by insects reduce reproductive output in *Aloe reitzii* var. *reitzii* (Asphodelaceae)

ABSTRACT

Seed predators are a major factor limiting plant fecundity, as they reduce seed set, seed viability and recruitment, having important effects on plant population dynamics and trait evolution in plants. Parasitoid wasps and phytophagous insects have been recorded in *Aloe* fruit and seeds, but very few studies have detailed their impact on the reproductive output of aloes. This study aimed to identify the main phytophagous and/or parasitoid insects predating on *Aloe reitzii* var. *reitzii* fruit and seeds and to investigate the effects of these insect predators and parasitoids on the aloe's reproductive output. Seven insect phytophages and parasitoids were reared from *Aloe reitzii* var. *reitzii* fruit and seeds from racemes in emergence boxes – two *Apenthecia* spp. (Drosophilidae), booklouse (Psocoptera) and four wasp species – most of which are associated with other aloes. The chalcid wasp *Eurytoma aloineae* comprised the majority of insect emergent abundance and is the predominant pre-dispersal seed predator of *Aloe reitzii* var. *reitzii.* Average fruit set was 75% and average percentage predated fruit per aloe was 10.62%. Average seed set per aloe was 22.74 seeds per fruit and the average number of seeds affected by predation was 5.09 seeds per fruit, with as much as 55% of seeds in an individual fruit predated on. The reproductive output of *Aloe reitzii* var. *reitzii* was thus greatly impacted by predation, which could have significant impacts on the fecundity and recruitment of its plants. This may significantly impact the persistence and genetic diversity of *Aloe reitzii* var. *reitzii* populations, many of which are fragmented due to habitat degradation.

KEYWORDS: *Apenthecia* sp.; *Eurytoma aloineae*; Parasitoid; Phytophages; Pre-dispersal seed predation

INTRODUCTION

Plant-animal interactions can be mutualistic, whereby animal-pollinated plants are dependent on fauna for reproduction and the plants provide food for the animals (Kearns *et al.*, 1998) in the form of nectar and pollen, or other rewards, such as oil (Fægri and van der Pijl, 1979). However, animals can also be antagonistic, causing damage to plants by removing plant tissue or by feeding on seeds (Kolb *et al.*, 2007). Compared to herbivory, in which plant tissue is only partially removed, seed predation can lead to complete loss of individual plants (Hulme and Benkman, 2002). Therefore, seed predation involves all levels of plant-animal interactions from individuals to plant communities (Janzen, 1971), and plays an important role in plant population demography (i.e., abundance and distribution; Kolb *et al.*, 2007), and imposes strong selective pressures on plants (Hulme and Benkman, 2002).

Compared to other plant structures, fruits and seeds are very attractive to insect predators and parasites (Sallabanks and Courtney, 1992). Fruits are particularly important in the reproductive cycle of frugivorous insects, which use fruits as a habitat and food source, as well as to mate and oviposit (Sallabanks and Courtney, 1992). Seeds are nutrient-rich and are a key food source for granivorous animals (Hulme and Benkman, 2002). Seed predators are often distinguished as pre-dispersal or post-dispersal seed predators (Janzen, 1971; Sallabanks and Courtney, 1992; Hulme and Benkman, 2002). Pre-dispersal seed predators feed on seeds on the parent plant prior to seed dispersal, and are characterized as “specialist” feeders that synchronize their life cycles based on seed availability (Hulme and Benkman, 2002). These predators mainly belong to insect orders Diptera, Lepidoptera, Coleoptera and Hymenoptera (Crawley, 2000). Additionally, some pre-dispersal seed predators may also be pollinators of their host plant, as observed in *Silene vulgaris* (Moench) Garcke (Pettersson, 1991). In *S. vulgaris*, female *Hadena* sp. moths feed on nectar and pollinate *S. vulgaris* flowers, but also oviposit their eggs into the flowers, whereby flowers and seed capsules provide food for their larvae (Pettersson, 1991). Post-dispersal seed predators feed on seeds after they are dispersed (Hulme and Benkman, 2002), and are more generalist herbivores, namely rodents, ants and birds (Crawley, 2000; Hulme and Benkman, 2002). Most studies, however, have been concerned with pre-dispersal seed predators, due to their considerable impact on plant fecundity.

Seed predators are a major factor limiting plant fecundity, as not only have they been shown to reduce seed set (Louda, 1982; Anderson, 1988, Vaughton, 1990; Zammit and Hood, 1996), but they also reduce seed viability (Anderson, 1988) and recruitment (Auld and Myerscough, 1986). They therefore affect population growth rate and number of individuals available for future generations (Kolb *et al.,* 2007). Therefore, pre-dispersal seed predation may have important effects on plant population dynamics and trait evolution in plants (e.g., flowering phenology and flower number, Kolb *et al.,* 2007). It is therefore important to recognize the diversity of insect taxa associated with fruits and seeds (Sallabanks and Courtney, 1992), and to quantify the impact of seed predation on plants within and among plant populations.

Insects play an important role in the pollination of aloes, as primary pollinators (Botes *et al.*, 2009b; Symes *et al.*, 2009; Hargreaves *et al*., 2012), co-pollinators, or secondary pollinators (Botes *et al.*, 2009a, 2009b; Hargreaves *et al.*, 2008, 2012; Patrick *et al*., 2018), but little is known about the role of insects as predators and their effect on the reproductive output of aloes. Recent studies on two *Aloe* species have shown that they can be a host to a variety of pre-dispersal predators — phytophagous insects and their associated parasitoids (viz., *A. pretoriensis* Pole Evans, van den Bosch *et al.*, 2019; *A. lettyae* Reynolds, Kremer-Köhne *et al.*, 2020). Predation of fruit and seeds by these phytophagous insects has been shown to considerably reduce the aloes' reproductive output (van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020), which may drastically impact aloe populations, especially fragmented populations or those present on 'habitat islands' (van den Bosch *et al.*, 2019). Prior to these two detailed studies, only the chalcid wasp *Eurytoma aloineae* Burks (Eurytomidae) had been reported as predating the seeds of seven aloe species, viz., *A. africana* Mill., *A. ferox* Mill., *A. globuligemma* Pole Evans, *A. lineata* (Aiton) Haw., *A. microstigma* Salm-Dyck, *A. striata* Haw., and *A. variegata* L. (Burks 1958; Prinsloo, 1980). Because of the impact of the phytophagous and/or parasitoid insects (mainly wasps and drosophilid flies) on the reproductive output of aloes, it is important to investigate the role of insects in aloe fruit and seed predation.

Aloe reitzii Reynolds var. *reitzii* (hereafter referred to as *A. reitzii*) is Near-Threatened, with habitats lost due to commercial timber plantations and populations increasingly threatened due to mining and quarrying development (Mtshali *et al*., 2018). In a previous study on *A. reitzii*, shriveled fruit were observed on 33% of plants (Payne, 2019), along with the presence of small insects in *A. reitzii* fruits and seeds (S. Payne, *pers. comm.*). Since insects promote fruit abortion by damaging fruits (Sallabanks and Courtney, 1992), predation of *A. reitzii* fruits and seeds may be widespread in *A. reitzii* populations. Therefore, this study aimed to identify and quantify the variety of phytophagous and/or parasitoid insects predating on fruits and seeds of *A. reitzii* using emergence boxes, and to investigate their impact on fruit set and seed set, i.e. the effect on its reproductive output.

MATERIALS AND METHOD

Study species and study site

The summer-flowering *Aloe reitzii* var. *reitzii* is endemic to the rocky slopes and grasslands of Mpumalanga, with localized distributions in Limpopo and Gauteng (Van Wyk and Smith, 2008). It is a stemless aloe (Figure 4.1), reaching a height of 1 m (Symes, 2017), with a rosette height (leaf height) of 66.6 cm and a mean rosette diameter of 60.6 cm (Payne, 2019). It is characterized by dark red flowers that yellow as they mature (Van Wyk and Smith, 2008). Mature plants can have up to six branches of racemes (Van Wyk and Smith, 2008), whereas young plants generally have a single raceme reaching up to 2 m in height (van Wyk and Smith, 2008; Symes, 2017). The study was conducted on Klipbankspruit Farm (25° 16’ 21.9’’ S 30° 3’14’’ E; ~1840 m a.s.l.), approximately 30 km north-west of Dullstroom, Mpumalanga, where *A. reitzii* grows in abundance on the rocky hillsides.

Figure 4.1. Mature, flowering *Aloe reitzii* var. *reitzii* plant at Klipbankspruit farm. Scale bar = 10 cm. Photograph by Jessica Minnaar.

Main insect predators/parasitoids

To determine the type and abundance of phytophagous and parasitoid wasps/insects present in *A. reitzii* fruit, a single fruiting raceme from each of 10 aloes was placed in an emergence box over a six-week period. It was placed in water to prevent drying of the fruit. Glass vials were placed onto funnels at the top of the emergence boxes, and vials were checked daily for emerging insects. All emergent insects collected were stored frozen. They were examined under a Zeiss dissecting microscope and photographed with an attached Axiocam MRc camera. Insects were identified as far as possible using field guides and by reference to samples/photographs from previous studies (viz., van den Bosch *et al.*, 2019), and sent to experts[1] to confirm identifications.

Effect of insect predators/parasitoids on fruit set and seed set

The number of fruits that were predated on by insects was counted by examining each fruit for holes, indicating the emergence of an insect. The number of aborted fruit, identified as shriveled fruit, was also noted. The total number of fruits (with and without emergence holes) produced by each aloe was also counted, and fruit set was determined by counting the percentage of flowers per raceme, represented by the number of pedicels present after flowers had fallen off (Symes *et al.*, 2009). The impact of fruit predation relative to the whole raceme (fruit utilization) was calculated as a proportion of flowers on the raceme that set fruit (van den Bosch *et al.*, 2019). Fruit set (whole and shrivelled), fruit utilization (%) and percent predated fruit were determined using the following equations:

$$\text{Fruit set (\%)} = \frac{\text{No. of fruit produced}}{\text{No. of total flowers per raceme}^{2}} \text{ x } 100$$

$$\text{Fruit utilization (\%)} = \frac{\text{No. of predated fruit}^{3}}{\text{No. of total flowers per raceme}^{2}} \text{ x } 100$$

$$\text{Predated fruit (\%)} = \frac{\text{No. of predated fruit}^{3}}{\text{No. of fruit produced}} \text{ x } 100$$

[2] Number of flowers represented by the number of pedicels present after flowers had fallen off.

[3] Number of fruits with emergence holes.

[1] Wasps: Simon van Noort (Iziko Museums of South Africa); Drosophilidae: Shane McEvey (Australian Museum Research Institute)

To assess the effect of insect predators/parasitoids on seed set of *A. reitzii*, the presence of holes in seeds (indicating the emergence of wasps), as well as the presence of larvae in the fruit were recorded. Ten fruit were collected from each raceme before racemes were placed into emergence boxes. Seed set was determined by first air drying each individual fruit at ambient temperature, and once the fruit had dehisced, the number of seeds in each of the 10 harvested fruits was counted to calculate the average number of seeds per fruit (Arena *et al.*, 2013). To determine the extent to which an individual *A. reitzii* plant was impacted by predation, fruit utilization and seed utilization (number of seeds per fruit impacted by predation) was extrapolated by multiplying by the average number of racemes across ten aloes, since the number of racemes per aloe used in this study was not recorded. Seed set, seed utilization and percent predated seeds were determined using the following equations:

$$\text{Seed set} = \frac{\text{No. of total seeds produced}}{\text{No. of fruit}}$$

$$\text{Seed utilization} = \frac{\text{No. of predated seeds}^{1}}{\text{No. of fruit}}$$

$$\text{Predated seeds (\%)} = \frac{\text{No. of predated seeds}^{1}}{\text{No. of seeds}} \text{ x } 100$$

[1] Number of seeds with holes present (indicating the emergence of wasps)

Data analysis

To determine the predominant insect fruit and seed predators in *A. reitzii*, the total abundance of each insect species that emerged from each aloe was calculated, as well as the total abundance of each insect across all aloes. The effect of fruit and seed predators on the reproductive output of *A. reitzii* was determined using the equations stipulated above.

To test if there is a correlation between fruit set and the number of impacted fruits by predators, simple linear regressions were used to compare: fruit set and percentage predated fruit (effect of parasites on fruit); fruit set and percentage predated seeds (effect of parasites on seed). Additionally, to test if there is a relationship between the number of fruits impacted by parasites and the number of seeds impacted, a simple linear regression of percent predated fruit and percent predated seed of *A. reitzii* was determined. All statistical analyses were conducted in R version 4.0.0. (R Core Team, 2020).

RESULTS

Main insect predators/parasitoids of A. reitzii

To infer the main insect predators/parasitoids of *A. reitzii*, emergence boxes were used to determine the abundance and type of insects found in fruits of *A. reitzii.* The first insects started emerging two days after being placed in emergence boxes. *Eurytoma aloineae* was the predominant insect to emerge (Figure 4.2A), contributing 89% of all insects that emerged (Figure 4.3). *Eurytoma aloineae* was also the most commonly occurring seed predator wasp across all the plants (89%; Figure 4.3) and comprised between 56% and 98% of all insects that emerged from each individual aloe in the emergence boxes (Figure 4.4). Other minor species that emerged were three wasp species: *Afrostilba* sp. (Figure 4.2B), a species of Torymidae (Figure 4.2C), and *Bracon* sp. (Figure 4.2D). However, these wasp species were not abundant, together only contributing 0.55% of insect emergence abundance (Figure 4.3, Figure 4.4). Two other flies also emerged from the fruit, both species of *Apenthecia* (Drosophilidae; Figure 4.2E, F), which together comprised 2% of total emerged insect abundance (Figure 4.3). Additionally, *Apenthecia* was also observed to be a floral visitor to *A. reitzii* (Chapter 3), most likely ovipositing its eggs in *A. reitzii* ovaries. An unidentified species of booklouse (Psocidae) was the next most common insect to emerge (Figure 4.2G), comprising 8.4% of emerging insects (Figure 4.3). However, the booklouse species comprised up to 39% insect abundance in an individual aloe (Aloe I; Figure 4.3). Overall, *A. reitzii* is host to three phytophagous insects, namely *E. aloineae* and the two *Apenthecia* species. The remaining wasp species, viz. *Afrostilba* sp., *Bracon* sp., and Torymidae sp., are parasitoids of phytophagous insects.

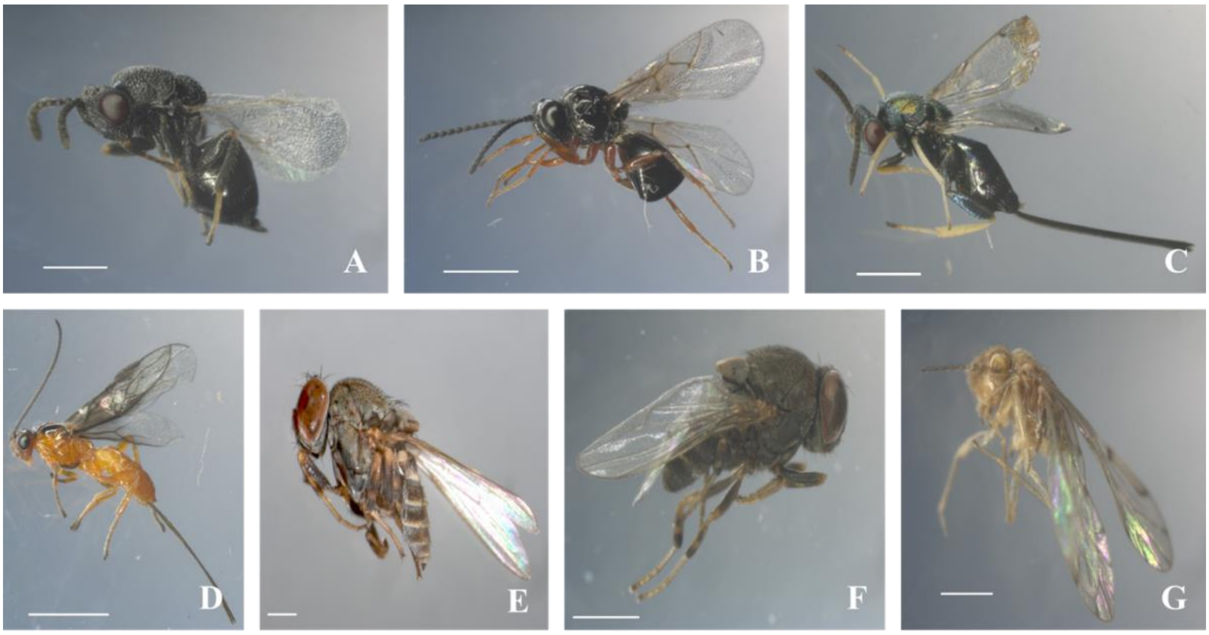

Figure 4.2. Insect parasites and their associated parasitoids reared from racemes of *Aloe reitzii* var. *reitzii* in emergence boxes: A) *Eurytoma aloineae* (Chalchilidae), B) *Afrostilba* sp. (Cynipoidea), C) Torymidae; D) *Bracon* sp. (Braconidae); E) *Apenthecia* sp.1 (Drosophilidae); F) *Apenthecia* sp. 2 (Drosophilidae) and G) Psocoptera (booklouse). Scale bars: A, E, G = 500 µm, B, F = 1 mm, C, D = 2 mm. Photographs by Jessica Minnaar.

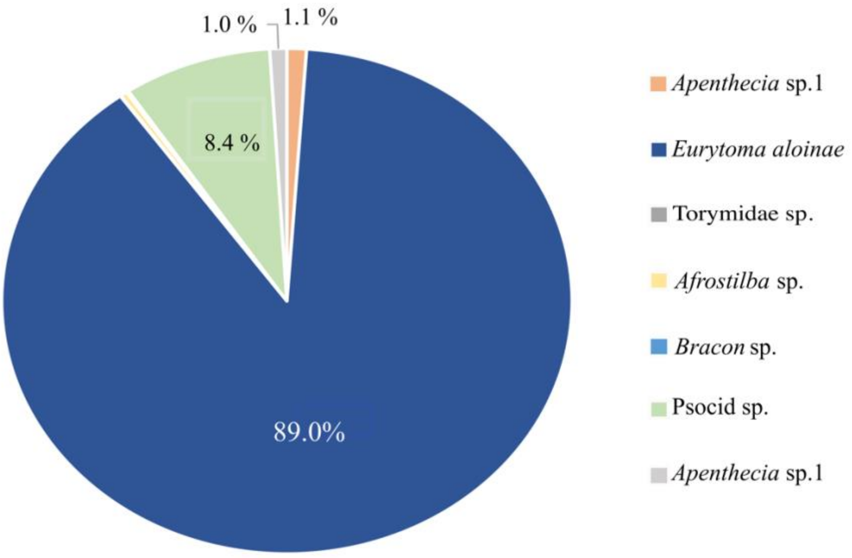

Figure 4.3. The total abundance of insects that emerged from *Aloe reitzii* var. *reitzii* fruit in emergence boxes, showing the total percent abundance of *Eurytoma aloineae* (dark blue), Psocoptera sp. (green) and *Apenthecia* spp. (light grey and orange). The total abundance of *Afrostilba* sp. (yellow), *Bracon* sp. (light blue), and Torymidae sp. (light grey) were too small to depict, comprising 0.37%, 0.14% and 0.05%, respectively.

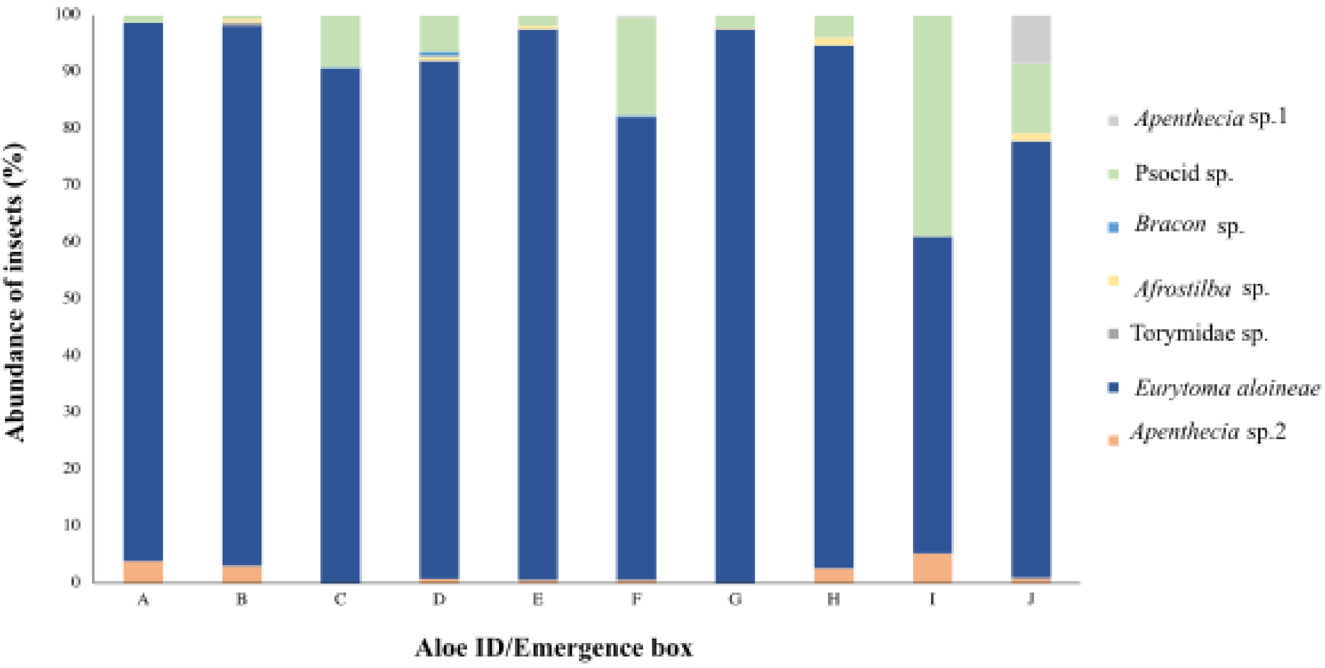

Figure 4.4. The proportion of insects that emerged fruits of *Aloe reitzii* var. *reitzii* racemes placed in emergence boxes (A – J); *Apenthecia* spp. (orange and light grey), *Eurytoma aloineae* (dark blue), Psocid sp. (green), Torymidae sp. (dark grey), *Bracon* sp. (light blue) and *Afrostilba* sp. (yellow).

Effect of predators/parasitoids on fruit set and seed set

Predated fruits were identified by having a distinct hole from which insects had emerged, with some fruits having two or three holes, with a maximum of five holes counted on a single fruit. Additionally, larval remnants (N = 14 larvae), possibly from emerged wasps/flies were present in 14 *A. reitzii* fruit. Where these larvae were present, fruits were shriveled, and no intact and/or fully developed seeds were found but left a dust-like deposit. From the 100 fruits used to determine seed set of *A. reitzii*, a total of 108 *E. aloineae* and nine drosophilid individuals were found that had not successfully emerged from the fruits and/or seeds. The maximum number of *E. aloineae* wasps found in a single fruit was 11.

To investigate the effects of insect predators/parasitoids on the fruit and seed set relative to individual racemes of *A. reitzii*, the impact of parasites on fruits (fruit utilization) and seeds (seed utilization) was determined. The average fruit set per raceme was 75.02 ± 2.18% and the average predated fruit utilization per raceme was 10.62 ± 1.07% (Table 4.1).

Fruit set and percentage predated fruit were not correlated (Rs = 0.4, *P* = 0.57; Figure 4.4A), nor did they significantly differ (β = -0.14± 0.24, *P* = 0.57). The average seed set per aloe was 22.74 ± 1.93 seeds per fruit and the average percentage of seeds affected by parasites (seed utilization) was 5.92 ± 0.56 seeds per fruit (Table 4.1). As much as 55% of the seeds were impacted by predation in an individual fruit, with some fruits having no seeds impacted by predation. Percentage of predated seeds was negatively correlated with fruit set (Rs = 0.6, *P* < 0.001), with significantly more impacted seeds found in aloes with a lower fruit set (β = 95.4 ± 21.17, *P* < 0.001; Figure 4.4B). Percentage predated fruit and seeds were not significantly correlated (Rs = 0.002, *P* = 0.91; Figure 4.4C), nor were they significantly different (β = 0.07 ± 0.67, *P* = 0.91). Individual aloes were significantly impacted by insect fruit and seed predators, as 47.47± 4.29% of fruit set and 23.69 ± 2.23 of seed set were impacted by seed predators.

Table 4.1. Fruit set, percent predated fruit, predated fruit set (fruit utilization; %), seed set (seeds per fruit), percent predated seeds (%) and predated seed set (seed utilization; seeds per fruit) in ten *Aloe reitzii* var. *reitzii* plants by phytophagous and/or parasitioid insects from emergence boxes. Mean and standard error (SE) and range are presented here.

	Mean ± SE	**Range**
Fruit set (%)[1]	75.02 ± 2.18	62.3 – 84.6
Predated fruit (%)	14.23 ± 1.50	6.6 – 21.5
Fruit utilization (%)	10.62 ± 1.07	4.5 – 15.4
Seed set[2]	22.74 ± 1.93	15.3 – 35.9
Predated seeds (%)	17.96 ± 2.83	15.3 – 29.3
Seed utilization	5.92 ± 0.56	3 – 8.2

[1] Only mature fruit used in calculations and fruit set includes predated fruit.

[2] Only fully developed seeds used in seed set calculations. Seed set calculations include predated seeds.

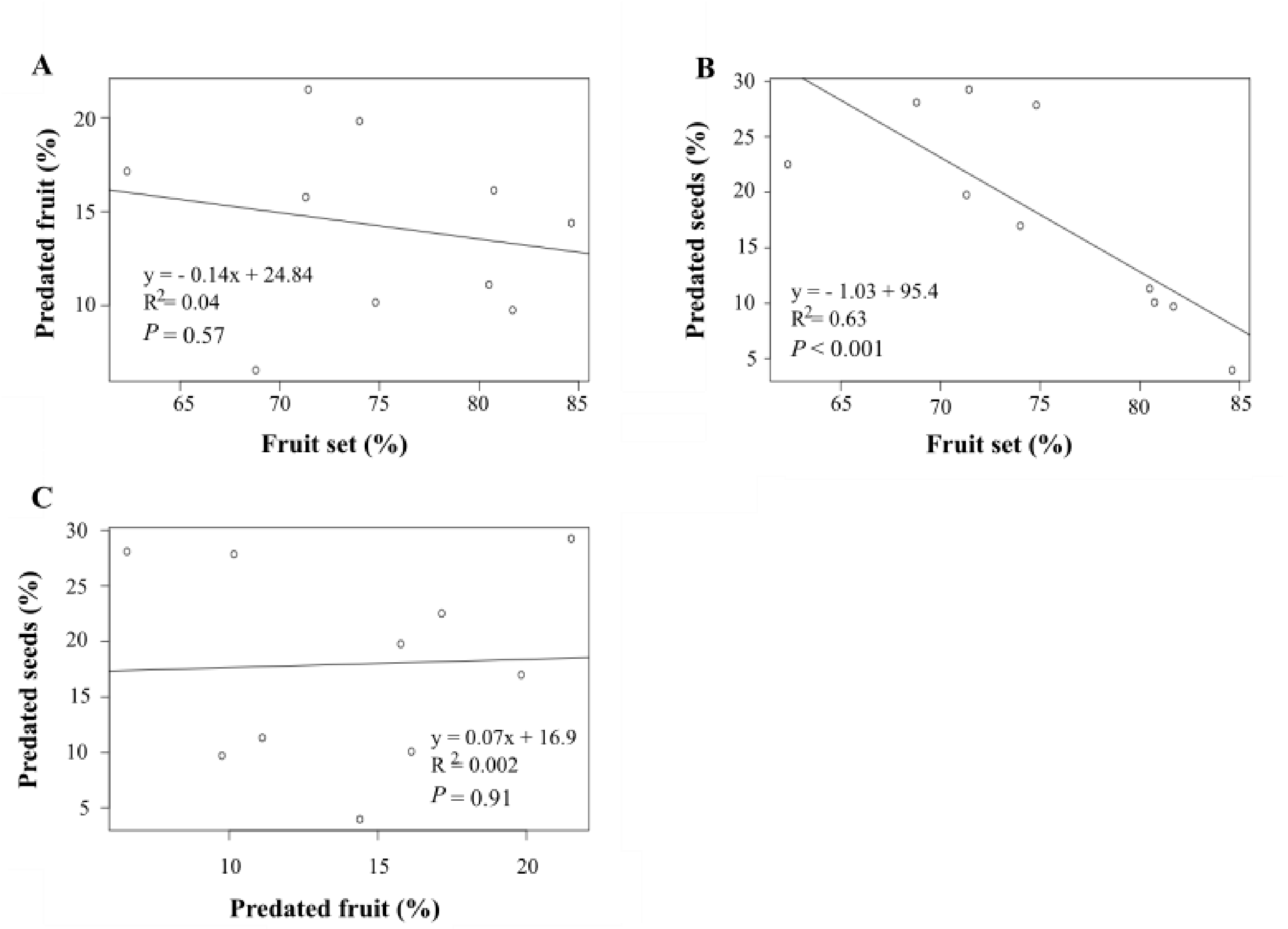

Figure 4.4. Correlations between (A) Percentage fruit set and percentage predated fruits, (B) percentage fruit set and percentage predated seeds and (C) percentage predated fruit and seeds.

DISCUSSION

The main insect predators and/or parasitoids of *A. reitzii* were investigated using emergence boxes. A total of seven insect phytophages and/or parasitoids were identified occurring in *A. reitzii* fruits and/or seeds. Four of the seven insect species emerging from *A. reitzii* fruits and/or seeds have been recorded associated with other aloes (Prinsloo, 1980; Bouček, 1983; van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020). As in other *Aloe* predation studies, the chalcid wasp, *E. aloineae*, is the most common phytophagous seed predator in *A. reitzii* fruit and seeds, comprising most of the insect emergence abundance. *Eurytoma aloineae* are phytophagous seed predators, stem feeders and gall-inducers, and parasitoids of a wide range of insects (Gates and Delvare, 2008; van Noort, 2021). *Eurytoma aloineae* is a common pre-dispersal seed predator of *Aloe* (Prinsloo, 1980). The chalcid wasp is associated with summer-flowering aloes (e.g., *A. lettyae*, Kremer-Köhne *et al.*, 2020), and is also common in many winter-flowering aloes (Burks, 1958; van den Bosch *et al.*, 2019). The presence of both *E. aloineae* and *Apenthecia* in *A. reitzii*, similar to other aloes (e.g., *A. pretoriensis*, van den Bosch *et al.*, 2019;

A. lettyae, Kremer-Köhne *et al.*, 2020), suggests a parasite-host relationship between *E. aloineae* and *Apenthecia* sp. in *A. reitzii* fruit. However, this relationship remains relatively unexplored and needs to be confirmed (van den Bosch *et al.*, 2019).

Although not highly abundant in *A. reitzii*, *Apenthecia* spp. flies were abundant in the other two aloes where they were quantified (van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020). *Apenthecia* spp. use *Aloe* flowers and fruits as breeding sites (Chassagnard *et al.*, 1997), and are host to various wasp species, such as *E. aloineae* (Bouček, 1983) and *Afrostilba* sp. (Kremer-Köhne *et al.*, 2020), thereby attributing to the variety of parasitoid wasp species in *A. reitzii.* Other wasp species reared from *A. reitzii* fruit, namely *Afrostilba* sp., *Bracon* sp., and Torymidae sp., are parasitoids of phytophagous insects. For example, *Afrostilba* sp. is a fly parasitoid, and is particularly associated with *Apenthecia* sp. in *Aloe* (Kremer-Köhne *et al.*, 2020), and leaf-mining Agromyzidae flies (van Noort *et al.*, 2015). *Bracon* sp. wasps are endoparasitoids of larvae of xylophagous and stem-boring Coleoptera and Lepidoptera, rarely feeding on Diptera and Symphyta (van Noort, 2021). Although not abundant in *A. reitzii*, *Bracon* sp. comprised 19% emergence abundance in *A. pretoriensis* (van den Bosch *et al.*, 2019), and may therefore also be a common parasitoid in *Aloe* species. Only a single individual of the wasp in the Torymidae was found in *A. reitzii* fruit. They are known ectoparasitoids or phytophagous insects of gall-forming insects (Cynipidae and Cecidomyudae) and primary parasitoids of hyperparasitoids of insect eggs and larvae of Diptera, Hymenoptera, Lepidoptera, Coleoptera or Coccoidea (Grissell, 1995; van Noort, 2021).

Psocoptera (psocids), the second most abundant insect to emerge from *A. reitzii* fruits, are booklice commonly found in bark, leaf litter and winter stores, that feed on lichen, fungi and plant-animal debris (Picker *et al*., 2004). Psocids were often observed crawling between flowers and fruits on *A. reitzii* racemes (J. Minnaar, *pers. obs.*), and possibly use flowers and/or fruits to oviposit their eggs. Although not parasitic (Picker *et al.*, 2004), some psocids are known to be insect predators (New, 1987), but they are most commonly found infesting grain and grain products (Sedlacek *et al.*, 1995). Since the study population of *A. reitzii* was located on a farm, psocids may be common, as they are known for their ability to opportunistically colonize and exploit environments in which they occur (New, 1987). They may be using the surrounding *A. reitzii* plants to live and reproduce, accounting for their high abundance in the *A. reitzii* fruit. However, the role of psocids in *A. reitzii* and their impacts on the reproductive output of *A. reitzii* needs to be further investigated.

Eurytoma aloineae were also common floral visitors to *A. reitzii*, suggesting a dual purpose at *A. reitzii* flowers: to feed on nectar and to oviposit their eggs in flowers and fruits, and/or feed on associated hosts (van den Bosch *et al.*, 2019). Most parasitoids are dependent on nectar from flowers or extra-floral nectar and honeydew for nourishment (Gess and Gess, 2014). Nectar, in particular, provides parasitoid wasps with nutrients essential to increase their longevity (Lee *et al.*, 2004), fecundity (Rose *et al.*, 2006), flight capacity (Wanner *et al.*, 2006), and improves their host-search capabilities (Lewis and Takasu, 1990; Patt *et al.*, 1999). Parasitic wasps are therefore able to save time and energy when feeding on plant resources associated with their host plant and/or prey species (Wäckers *et al.*, 2008). Wasps may therefore have a dual purpose at *A. reitzii* flowers; firstly to drink nectar for nutrients, and secondly to use *A. reitzii* fruits and flowers to oviposit their eggs on host species (van den Bosch *et al.*, 2019).

The impact of phytophagous insects and/or parasitoids on the reproductive output of *A. reitzii* was measured by investigating impacts of these insects on fruits and seeds of *A. reitzii*. Phytophagous insects, such as *Apenthecia* spp., and predispersal seed predators *E. aloineae*, cause considerable reductions in seed set and fruit set of *A. reitzii*, impacting on average 47% of fruits in an individual plant and as much as a 55% of seeds in an individual fruit. A similar pattern was observed in two other aloe species in which predator/phytophage/parasitoid impact was quantified (van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020). Pre-dispersal seed predators cause considerable reduction in seed set of plants (Auld and Myerscough, 1986; Zammit and Hood, 1986; Anderson, 1988; Vaughton, 1990), reducing fecundity and impacting plant recruitment (Louda, 1982; Auld and Myerscough, 1986). As noted above, *A. reitzii* is Near-Threatened, with approximately 30% of its habitat irreversibly modified due to habitat loss and degradation (Mtshali *et al.*, 2018), resulting in fragmented populations of *A. reitzii*. Since more seeds are impacted by predation compared to fruits, pre-dispersal seed predators significantly impact fecundity, which could have a considerable impact on plant recruitment. Therefore, pre-dispersal seed predators in *A. reitzii* may impact persistence of *A. reitzii* populations. However, a population demographic study is needed to show the impact of pre-dispersal predators on the persistence of *A. reitzii* populations.

Conclusion and future work

The presence of phytophagous insects and/or parasitoids in *A. reitzii* fruits and seeds considerably reduce the reproductive output of *A. reitzii*. *Eurytoma aloineae* is the main pre-dispersal seed predator in *A. reitzii,* which together with other phytophagous insects, viz. *Apenthecia,* cause substantial reductions in healthy fruit set and seed set. However, the relationship between parasitoids and their associate host insects in *Aloe*, such as between *E. aloineae* and *Apenthecia*, remains unexplored. To determine the relationship between parasitoids and their hosts in *A. reitzii,* the presence of parasitoids and phytophages during all flowering stages could be investigated (e.g., van den Bosch *et al.*, 2019), as well as during the different fruiting stages. Psocids were the second most common insect to emerge from *A. reitzii* fruit, but their impact on the reproductive output of *A. reitzii* is unknown. Additionally, it is unknown whether psocids oviposit eggs in flowers of *A. reitzii* or on raceme stalks. Therefore, the abundance of psocids at *A. reitzii* plants during flowering, and whether they are parasitoids and/or phytophages of *A. reitzii* should be further investigated.

Proximity to other plants increases the number of *Aloe* plants impacted by predation (van den Bosch *et al.*, 2019), and plants further from their conspecifics suffer more fruit predation (Metcalfe and Kunin, 2006). To determine whether similar patterns are observed in *A. reitzii*, the proportion of *A. reitzii* plants impacted by predation relative to their proximity to flowering conspecifics and other plant species should be investigated. More seeds are impacted by predation compared to fruits, reducing fecundity and recruitment of *A. reitzii* plants, which may significantly impact *A. reitzii* plant population dynamics. Since *A. reitzii* populations are increasingly impacted by habitat degradation (Mtshali *et al.*, 2018), more populations may become fragmented. Therefore, pre-dispersal seed predation could drastically impact *A. reitzii* populations, especially those in “habitat islands” (van den Bosch *et al.*, 2019). Therefore, to determine the extent of parasitism in different *A. reitzii* populations, the impact of phytophagous insects and parasitoids of *A. reitzii* should be investigated and compared among *A. reitzii* populations. Although the presence of parasitoids has been recorded in a variety of *Aloe* species, only a few studies have detailed the impact of parasitoids and phytophages on the fruit and seeds in *Aloe* (viz. van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020) thus more detailed studies of parasitism in *Aloe* fruit and seeds are necessary to determine how widespread predation and parasitism are in the genus.

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APPENDIX A

Table. Fruit set (%) and seed set (number of seeds per fruit) of *Aloe reitzii* var. *reitzii*, and fruit utilization (impact of fruit predation relative to the whole raceme; %), predated fruit (%), number of predated seeds relative to the whole raceme (seed utilization; seeds per fruit) and predated seeds (%) by phytophagous and parasitic insects recorded from a single raceme of ten individual *Aloe reitzii* var. *reitzii* plants.

Aloe	**Fruit set (%)**	**Fruit utilization (%)**	**Predated fruit (%)**	**Seed set**	**Seed utilization**	**Predated seeds (%)**
A	71.3	11.2	15.77	22	6	19.77
B	62.3	10.7	17.14	26	6.9	22.52
C	74.8	7.6	10.16	15.3	6.8	27.86
D	68.8	4.5	6.56	15.9	6.8	28.1
E	74	14.7	19.82	35.9	8	16.99
F	81.7	8	9.76	17.7	6	9.71
G	71.4	15.4	21.5	27.3	8.2	29.26
H	80.7	13	16.13	23.4	4.1	10.07
I	84.6	12.2	14.39	21.4	3	3.97
J	80.5	8.9	11.11	22.5	3.7	11.32

CHAPTER FIVE

GENERAL OVERVIEW

Aloe reitzii var. *reitzii* (hereafter referred to as *A. reitzii*) has floral traits characteristic of an ornithophilous pollination syndrome, as shown by its large floral display of red-orange flowers (Van Wyk and Smith, 2008), copious nectar production (Symes, 2017; Payne, 2019) and long floral tubes (~45 mm; Chapter 3). Consistent with their floral traits, birds are primary pollinators of many ornithophilous aloes (e.g., Johnson *et al.*, 2006; Botes *et al.*, 2009; Hargreaves *et al.*, 2012), but bees also play a role in their pollination (Botes *et al.*, 2008, 2009; Hargreaves *et al.*, 2008, 2012). Both birds and bees were previously observed as abundant visitors to *A. reitzii* flowers (Symes, 2017; Payne, 2019), but their role in the reproductive ecology of *A. reitzii* had not been investigated. This study aimed to investigate: i) the reliance of *A. reitzii* on pollinators for reproduction by evaluating the breeding system of *A. reitzii*; ii) infer the relative roles of birds and insects in its reproductive biology –notably in effective pollination of *A. reitzii* flowers (Figure 5.1). Since Cape Weavers (*Ploceus capensis*) and Malachite Sunbirds (*Nectarinia famosa*) were both abundant visitors at *A. reitzii* flowers (Symes, 2017; Payne, 2019), the relative importance of generalist and specialist nectarivore bird species in the pollination of *A. reitzii* was also assessed. Lastly, the impact of insect predation and parasitism on fruit and seed set in this aloe was investigated, adding to the growing literature on this previously unstudied aspect of aloe ecology.

Breeding system of A. reitzii

The breeding system of *Aloe reitzii* was investigated to determine whether *A. reitzii* is self-incompatible and thus reliant on pollinators for outcrossing, or whether it is self-compatible and capable of autonomous self-pollination. The results clearly indicate that *A. reitzii* is self-compatible; this has only been reported in a few island-endemic *Aloe* species (e.g., *A. divaricata*, Ratsirarson, 1995; *A. mayottensis*, Pailler *et al.*, 2002) and only one South African aloe, *A. thraskii* (Patrick *et al.*, 2018). Similar to the self-compatible *A. thraskii* (Patrick *et al.*, 2018), *A. reitzii* is not capable of autonomous self-pollination, therefore requiring pollinators to transfer pollen between flowers within a raceme and between racemes on a plant. The large floral display, combined with its self-compatibility, makes *A. reitzii* susceptible to geitonogamous selfing, which could lead to inbreeding depression (de Jong *et al.*, 1993; Harder and Barrett, 1996). However, like many aloes, *A. reitzii* has evolved floral traits that reduce the probability of geitonogamous selfing, namely protandry, herkogamy and flowers that mature acropetally on the raceme (Botes *et al.*, 2008; Hargreaves *et al.*, 2012).

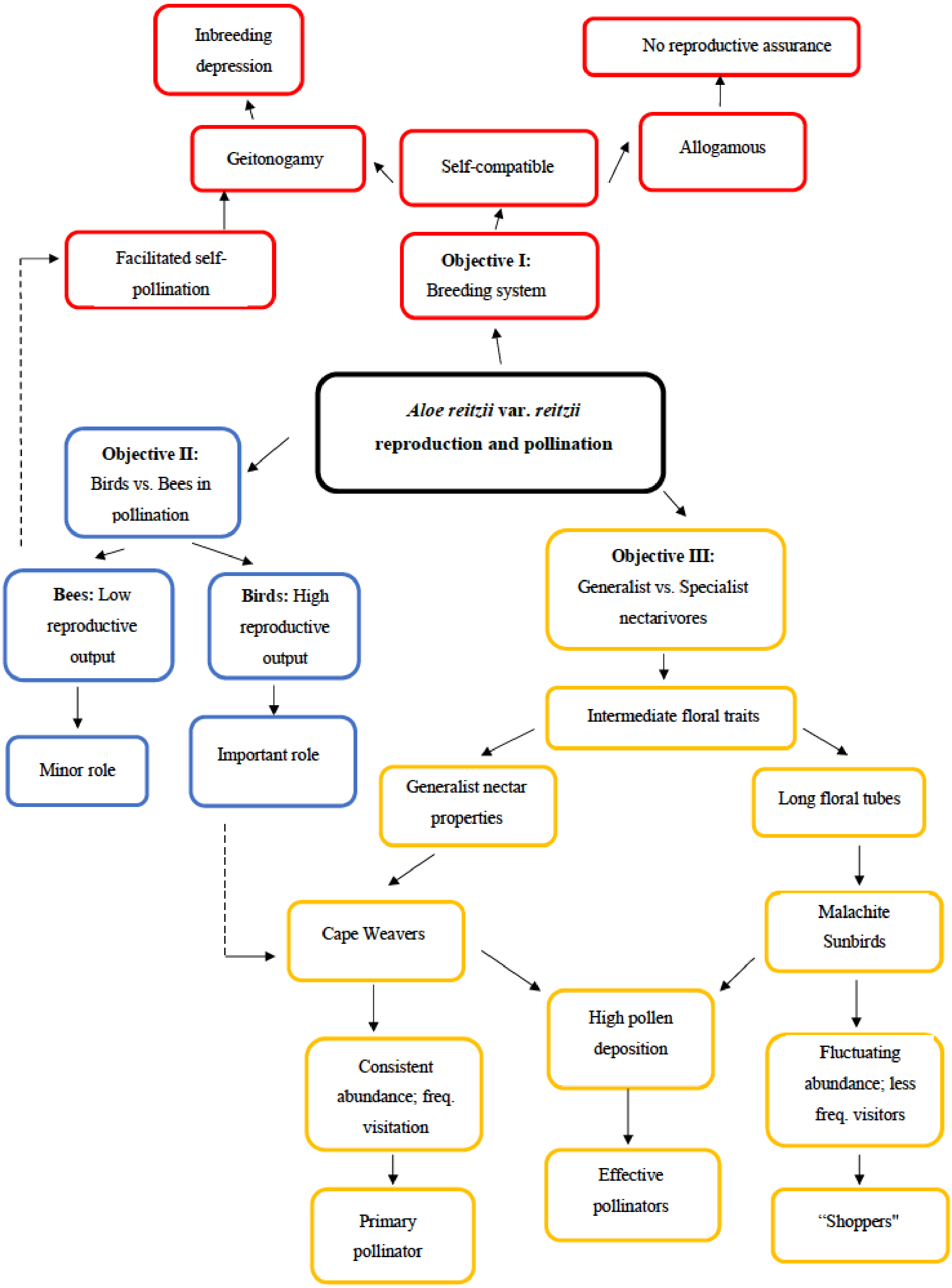

Figure 5.1. A broad synthesis of the results of the study. Findings of the study are based on each objective, with colours denoting different objectives (red = objective I; blue = objective II and yellow = objective III). Dashed arrow represents a link between findings for each objective.

These adaptive traits reduce levels of self-pollination by promoting efficient pollen dispersal (Barrett, 1998). *Aloe reitzii* could have a mixed-mating system, common in self-compatible plants (Lloyd, 1992), due to pollinator-mediated selfing by bees, and outcrossing via birds. Self-compatibility in *A. reitzii* is suggested here to have evolved due to its restricted distribution, in which smaller and fragmented populations experience pollinator limitation (Eckert *et al*., 2009; Busch and Delph, 2012).

Pollination of Aloe reitzii

Aloe reitzii is primarily pollinated by birds, which is consistent with its ornithophilous floral traits. Although highly abundant visitors, bees play a minor role in the pollination of *A. reitzii*, and likely facilitate geitonogamous self-pollination, accounting for the reduced fecundity observed in bird exclusion treatments. The abundance of Cape Weavers at *A. reitzii* has remained relatively consistent over multiple years (2014, 2017 and 2020), while that of Malachite Sunbirds has fluctuated. Therefore, Malachite Sunbirds are suggested to be "shoppers" at *A. reitzii* flowers, only increasing in abundance in response to increasing food availability. A similar pattern for Malachite Sunbirds has been observed in winter-flowering aloes (e.g., *A. ferox*, Forbes *et al*., 2009; Kuiper e*t al*., 2015). Since comparably high pollen loads were present on Cape Weavers and Malachite Sunbirds, both nectarivores are suggested to be effective pollinators of *A. reitzii.* However, due to their consistently high abundance and frequent visitation to *A. reitzii* flowers, Cape Weavers are deduced to be the primary pollinators of *A. reitzii*.

Although *Aloe* floral traits are used to infer whether aloes have a specialist or generalist bird pollination syndrome (Botes *et al*., 2008), the intermediate floral traits displayed by *A. reitzii* facilitate effective pollination by both generalist and specialist nectarivores. Thus, the long floral tubes and generalist nectar properties of *A. reitzii* flowers enable effective pollination by Cape Weavers and Malachite Sunbirds. Malachite Sunbirds have previously been shown to be effective pollinators of short-tubed aloes with dilute nectar production (e.g., *A. kraussii* and *A. ferox*, Hargreaves *et al.*, 2012), hence floral traits in aloes are not always a true indicator of primary bird pollinators. Correlations between bill morphology (shape and length) and floral traits have commonly been used to interpret whether birds are effective pollinators of specific plants (Botes *et al.*, 2008). However, despite its long floral tubes, the short-billed Cape Weavers are still able to effectively pollinate *A. reitzii* flowers. Cape Weavers feed on *A. reitzii* nectar by inserting their head into *A. reitzii* flowers, resulting in pollen mainly being deposited on their throats. In comparison, Malachite Sunbirds feed by inserting their long bills into the perianth tubes, while the strongly exserted filaments brush pollen onto their throats. Thus, the feeding behaviour of both bird species enables effective pollen transfer and cross-pollen deposition onto *A. reitzii* stigmas, which is made easier by the strongly exserted stamens and styles of *A. reitzii* flowers. Therefore, bill morphology and floral traits cannot solely be used to determine effective pollinators of ornithophilous aloes, and other factors such as birds' feeding behaviour should also be considered.

Fruit and seed predation in Aloe reitzii

A variety of insect phytophages and parasitoids were reared from *A. reitzii* fruit and seeds from racemes in emergence boxes, most of which are also associated with other aloes (e.g., wasp species *Eurytoma aloineae*, *Afrostilba* sp, *Bracon* sp., and drosophilid *Apenthencia* spp.). *Eurytoma aloineae*, commonly associated with seed predation in aloes (Burks, 1958; van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020), comprised the majority of insect emergence abundance and is the predominant pre-dispersal seed predator in *A. reitzii*. The phytophagous drosophilid, *Apenthecia* sp., also common in fruit of other aloes (e.g., *A. pretoriensis*, van den Bosch *et al.*, 2019; *A. lettyae*, Kremer-Köhne *et al.*, 2020), and the seed predator *E. aloineae* caused considerable reduction in the reproductive output of *A. reitzii*. Since more seeds were impacted by predation compared to fruit, pre-dispersal seed predation in *A. reitzii* reduces fecundity of plants which may have significant impacts on populations, especially fragmented populations resulting from habitat loss and degradation. Therefore, fruit and seed predation in *A. reitzii* could impact the persistence of *A. reitzii* populations but further investigation is required by conducting a population demographic study.

FUTURE RECOMMENDATIONS

Due to the combined effects of self-compatibility and pollinator-mediated selfing in *A. reitzii*, geitonogamy is unavoidable and *A. reitzii* populations may experience high levels of inbreeding. Future studies on *A. reitzii* should therefore investigate the prevalence of inbreeding in *A. reitzii* populations by measuring genetic variability within and among *A. reitzii* populations. Genetic variability can be measured using genetic diversity parameters and compared among *A. reitzii* populations, such as expected heterozygosity, proportion of polymorphic loci, number of alleles, genetic diversity (e.g., Nei's genetic diversity statistics; Hamrick and Godt, 1996) and inbreeding coefficients (Hamrick and Godt, 1996; Aguiler *et al.*, 2008; Duffy *et al.*, 2020). Additionally, inbreeding and outcrossing rated in populations can be estimated using codominant markers, such as allozymes, microsatellites or AFLPs (Keller and Waller, 2002; Galloway *et al.*, 2003; Jarne and David, 2008; Duffy *et al.*, 2020).

Since pollen brushed onto the sides of the bags covering the aloe racemes in the bagged treatments testing for autonomous selfing, wire frames should be added to bags to prevent any contact with open flowers in future studies (e.g., Symes and Nicolson, 2008; Arena *et al.*, 2013). To prevent any insects from being present in bagged treatments or crawling into bagged treatments, racemes should be checked carefully for any insects prior to bagging, and tape should be used instead of string/ribbon to secure bags to racemes. This is because the string may loosen over time, allowing tiny insects to crawl up the peduncle and access the flowers. Compared to *A. retizii*, self-compatible *A. thraskii* did not suffer reduced fecundity from self-pollen (Patrick *et al.*, 2018), thus pollen discounting and ovule discounting from self-pollen in *A. reitzii* should be quantified to determine its effects on fecundity for the self-compatible *A. reitzii*. Both honeybees and sweat bees carry high *A. reitzii* pollen loads and likely cause geitonogamous self-pollination. Therefore, to further investigate the role of bees in facilitating self-pollination in *A. reitzii*, the outcrossing rates and inbreeding depression coefficients can be compared between birds and bees (e.g., Duffy *et al.*, 2020).

Self-compatibility in *A. reitzii* may have evolved due to the aloe's restricted distribution and a similar investigation of the breeding system of the rare *A. reitzii* var. *vernalis* may provide insight into life history traits that may have resulted in self-compatibility and/or self-incompatibility in these varieties that both have restricted distributions. Since habitat fragmentation can cause shifts in mating patterns towards increased selfing (Aguiler *et al.*, 2008) due to reduced pollinator abundance and subsequent reduction in outcross pollen (Eckert *et al.*, 2009; Busch and Delph, 2012), the breeding systems of fragmented populations should be compared, as well as genetic variability within and among fragmented *A. reitzii* populations.

To determine whether bird species increase in abundance at *A. reitzii* populations in response to a copious nectar supply, bird species richness and the number of flowering plants should be investigated over successive flowering periods (e.g., Symes *et al.*, 2008; Forbes *et al.*, 2009; Kuiper *et al.*, 2015). This would also indicate whether there are changes in bird communities in response to the flowering of *A. reitzii*. The roles of the Greater Double-Collared Sunbirds (*Cinnyris afer*) and Amethyst Sunbirds (*Chalcomitra amethystina*) in the pollination of *A. reitzii* should be further investigated, as both are visitors to *A. reitzii*. To determine whether these specialist nectarivores are effective pollinators of *A. reitzii*, Amethyst Sunbirds and more individuals of Greater Double-Collared Sunbirds should be swabbed for pollen to assess *A. reitzii* pollen loads and main sites of pollen deposition. Similar to observations by Payne (2019), moths were found to be frequent night-time visitors and possibly probe the flowers. Their role in the reproductive ecology of *A. reitzii* should be explored by identifying moth visitors, assessing their behaviour at the aloes, quantifying night-time nectar production and floral scent, as well as carrying out night-time exclusion experiments to determine the effect of moths on the reproductive output of *A. reitzii*.

Although *Apenthecia* sp. is host to wasp *Afrostilba* species in *Aloe* (Kremer-Köhne *et al.*, 2020, the relationship between *E. aloineae* and *Apenthecia* sp. in *Aloe* fruits and seeds remains uncertain. Therefore, to determine the relationship between hosts and parasites in *A. reitzii*, the presence of phytophages and parasitoids in all flowering stages (e.g., van den Bosch *et al.*, 2019) and fruiting stages should be investigated. Psocids were the second most abundant insect to emerge from *A. reitzii*, but their role in *A. reitzii* is unknown. Therefore, whether psocids oviposit their eggs in *A. reitzii* flowers and/or fruit needs to be determined and their impact on the reproductive output of *A. reitzii* should be further investigated. Plants further away from conspecifics suffer greater fruit predation (Metcalfe and Kunin, 2006), thus the proportion of *A. reitzii* plants impacted by predation relative to their flowering conspecifics should be explored. *Aloe reitzii* populations are increasingly threatened by habitat loss and degradation (Mtshali *et al.*, 2018), resulting in fragmented populations. Therefore, the extent of parasitism among *A. reitzii* populations should be investigated. Few studies have detailed the effects of parasitoids and phytophages on the reproductive output in *Aloe* (viz. van den Bosch *et al.*, 2019; Kremer-Köhne *et al.*, 2020), therefore more detailed studies are necessary to determine how widespread fruit and seed predation and parasitism are in the genus.

Concluding remarks

In conclusion, *A. reitzii* is dependent on birds for pollination and successful reproductive output which is consistent with its ornithophilous traits. Since *A. reitzii* is self-compatible and susceptible to inbreeding depression, future studies on *A. reitzii* should focus on genetic variability within and among *A. reitzii* populations as it will be useful in determining the impacts of habitat fragmentation on this already restricted species, as well as the variety *Aloe reitzii* Reynolds *var. vernalis*. This may aid conservation efforts as the impacts of habitat loss and subsequent fragmentation of *A. reitzii* populations can be quantified. Since the same *A. reitzii* population has been investigated in all studies on the species, other *A. reitzii* populations should be investigated to infer whether similar patterns are observed, especially those concerning the composition and abundance of bird visitors to *A. reitzii*.

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