Pollination ecology and the functional significance of unusual floral traits in two South African stapeliads

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ABSTRACT

Carrion and dung mimicking plants often exhibit unusual floral traits which are believed to attract necro- and coprophagous insects as pollinators. Our understanding of these unusual traits and their functions is very limited. Stapeliads (Apocynaceae: Asclepiadoideae: Stapeliinae) are a monophyletic group of some 400 species of stem-succulent plants, many of which emit foul odours and exhibit unusual morphological traits that have anecdotally been assumed to represent adaptations to enhance the flowers' resemblance to carrion or dung. This study looked at the pollination biology of two stapeliads, *Orbea variegata* and *Stapelia hirsuta* var. *hirsuta*, and explored the functional significance of some of the floral traits commonly associated with carrion or dung mimicking flowers. Further, odours emitted by both species were compared to the odours of putative models to explore the chemical basis for the assumed mimicry.

Orbea variegata attracted flies from the families Muscidae, Calliphoridae and Sarcophagidae (at sites near Scarborough and Clifton, Western Cape) and individuals from each of these families were found carrying pollinia. The scent of *O. variegata* flowers was found to be dominated by dimethyl disulphide, dimethyl trisulphide as well as phenol. The presence of both these compounds suggests mimicry of both carrion and dung, although an ANOSIM analysis indicated that the odour of *O. variegata* shared more similarities with dung. This suggests that *O. variegata* is fairly generalist and explains the attraction of various flies that are associated with carrion or faeces by the flowers of this species. In experiments testing the importance of black versus yellow colouring and the importance of patterning, flies were found to prefer black coloured models in the presence of *O. variegata* odour, whereas the presence and size of blotching on the corolla lobes had no significant effect on fly visits. The colours of the black blotching and yellow of the corolla lobes showed minimal chromatic contrast when interpreted using the Troje (1993) fly vision model, although background rocks showed chromatic contrast, suggesting flies can distinguish between the flowers and the background. In an experiment testing the importance of odour for attracting flies, significantly fewer flies were able to locate concealed flowers compared to visible flowers, suggesting an important role for visual cues for flies to locate the odour source.

Stapelia hirsuta var. *hirsuta* was found to exhibit two floral colour morphs at Swellendam (Western Cape). The yellow morph was rarer than the maroon morph. These flowers attracted flies belonging to the Muscidae, Calliphoridae and Sarcophagidae families, although only Calliphoridae and Sarcophagidae were found to carry pollinia. The odour composition of these two morphs differed

slightly, where the odour of the maroon morph was dominated by dimethyl disulphide, dimethyl trisulphide and *p*-cresol and the yellow morph was dominated by dimethyl disulphide, dimethyl trisulphide and limonene. The ANOSIM analysis of odours emitted by *S. hirsuta* var. *hirsuta* in relation to that of various fly oviposition substrates suggested that these flowers are dung mimics rather than carrion mimics, although the presence of sulphides suggests possible mimicry of both. The yellow morph had higher fly visitation rates than the maroon morph. In experiments testing the role of floral trichomes, the removal of floral trichomes significantly decreased the visitation rates to the flowers, as well as the amount of time visitors spend on the flowers. Again, visual cues were shown to be of importance, as visible flowers received more visits than concealed flowers. Analysis of colours of different floral morphs, using the Troje (1993) fly vision model, suggests that flies cannot perceive chromatic colour differences between morphs.

In these studies, I have shown that *O. variegata* and *S. hirsuta* var. *hirsuta* are visited and pollinated by carrion associated flies, and the flowers emit odours associated with both carrion and dung. This work sheds light on some of the floral features that are often associated with carrion and dung mimicry by flowers and the roles they play in the attraction of flies.

PREFACE

The research described in this thesis was carried out at the School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg), from March 2015 to March 2017, under the supervision of Dr Adam Shuttleworth and the co-supervision of Prof. Steven Johnson (University of KwaZulu-Natal) and Prof. Sue Nicolson (University of Pretoria).

The work presented in this thesis represents the original work of the author and has not been otherwise submitted in any other form for any degree or diploma to any other University. Where use has been made of the work of others, this has been duly acknowledged in the text.

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COLLEGE OF SCIENCE AND AGRICULTURE
DECLARATION 1 – PLAGIARISM

I, Marc du Plessis, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
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Marc du Plessis
March 2017

ACKNOWLEDGEMENTS

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CHAPTER 1

Introduction

THE IMPORTANCE OF POLLINATORS IN SHAPING FLORAL TRAITS

The role of pollinators in shaping floral forms was first suggested by Darwin in the late 1800s (Darwin 1862; Darwin 1877) and this idea now forms the broad paradigm for much of the contemporary research on floral diversification. In other words, floral traits may often be interpreted as adaptations to the morphology and behaviour of pollinators that exist within the same area or habitat (Fenster et al. 2004). This micro-evolutionary concept of pollinator mediated evolution was later developed by Grant & Grant (1965) and Stebbins (1970), into a broad model of pollinator-mediated evolution driving floral diversification as plant populations encounter novel pollinators in different areas (this has subsequently been dubbed the "Grant-Stebbins" model (Johnson 2006; Johnson 2010)). This model is thus based on the notion that pollinators differ in functional morphology and have dissimilar floral preferences and geographic distributions, and this variability would likely result in pollinators applying divergent selection pressures on floral forms in allopatric populations (e.g. Newman et al. 2012). This increased appreciation for the role of pollinators in driving the evolution of floral forms has also enhanced our understanding of pollination syndromes.

In their modern form, pollination syndromes are described as patterns of convergent floral traits associated with particular pollinator types. However, the idea of pollination syndromes was not developed in an evolutionary context, but was originally developed as a means to classify floral diversity (Fenster et al. 2004; Johnson & Steiner 2000). These different forms (shapes and sizes) that the flowers exhibit are believed to produce sets of pollination syndromes, where specific pollinator features can be associated with the floral forms (Chittka et al. 2001; Faegri & van der Pijl 1979; Fenster et al. 2004; Hoballah et al. 2007; Vogel 1954). This concept was originally developed by the Italian botanist Federico Delpino in the late 1800s. Delpino (1868 – 1875) suggested that flowers can be classified in two ways, either where floral morphology could be a result of selection from visits by specific animals or classification merely by the animals that act as pollinating agents (Waser et al. 1996). This concept by Delpino (1868 – 1875) was then later developed into its modern form in the 1950s by Vogel (1954) and later by Faegri & van der Pijl (1979) (Johnson & Steiner 2000). The

contemporary concept of pollination syndromes is based on the assumption that pollinators can be placed in functional groups, regardless of species, based on shared morphologies, behaviours and preferences (Fenster et al. 2004). These functional groups of pollinators would be expected to exert similar selection pressures resulting in the observed patterns of floral convergence (Johnson & Steiner 2000; Fenster et al. 2004; Waser et al. 1996). An example of convergent floral traits resulting from selection by a functionally similar group of pollinators could be features such as long and narrow corolla tubes and volatile emission at night, which is associated with pollination by nocturnal hawkmoths (Ando et al. 1995; Hoballah et al. 2005; Johnson & Steiner 2000; Stuurman et al. 2004). Some of the functional pollinator groups that are traditionally associated with syndromes could include the following (from Fenster et al. 2004): long and short tongued bees which make up a pollen collecting functional group, long tongued bees and flies that could make up the long tongued nectar feeding group, and short tongued bees and flies that could make up a pollen feeding or nectar feeding functional group.

Two well-known pollination syndromes are: ornithophily (bird pollination) and chiropterophily (bat pollination), where Martén-Rodríguez et al. (2009) were able to provide support for these pollination syndromes using plants from the tribe Gesnerieae. They found that flowers that are bat pollinated corresponded in their floral floral traits to those described in over 700 bat pollinated species (Martén-Rodríguez et al. 2009). Some of these traits included: floral morphologies that facilitate access to nectar, open corollas to facilitate detection by echolocation and exposed floral displays to enhance accessibility (Martén-Rodríguez et al. 2009; Sanmartin-Gajardo & Sazima 2005). Further, Martén-Rodríguez et al. (2009) also found support for the hummingbird pollination syndrome, which can often be predicted by traits such as: tubular, red or yellow diurnal flowers with dilute nectar. This evidence largely demonstrates the importance of pollinator-mediated selection in floral diversification.

SAPROMYIOPHILY AND CARRION/FAECAL MIMICRY

Sapromyiophily is, in a very broad sense, defined as pollination by carrion and dung associated insects (various fly and beetle families), however, a more precise definition would include pollination by insects attracted to decaying organic material (Beaman et al. 1988; Chen et al. 2015; Davis et al. 2008; Jürgens & Shuttleworth 2015; van der Pijl 1961). The idea of a syndrome of sapromyiophily was first conceived by Delpino (1868-1875), originally called "sapromyiophilae" (carrion fly flowers), and it was further developed by Vogel (1954) as his "myophilous style". Vogel (1954) outlined a set of traits

of his "myophilous style", including exposed nectar, foetid odour, and a glossy or dull, warty surface with cilia often present (Jürgens & Shuttleworth 2015). This idea was further expanded by van der Pijl (1961), where he described a similar set of traits for his "syndrome of sapromyiophily", but also included the regular presence of filiform appendages, dark spots, and motile hairs. Plants that are classified in this syndrome are found in a diverse range of unrelated angiosperms, although they are particularly prevalent in some groups (Chen et al. 2015; Jürgens et al. 2006; Vereecken & McNeil 2010). Some of the families abundantly showcasing sapromyiophily include Apocynaceae, Aristolochiaceae, Sterculiaceae, Rafflesiaceae and Hydnoraceae in the dicots, and Araceae, Burmanniaceae and Orchidaceae in the monocots (van der Pijl 1961).

The syndrome of sapromyiophily appears to be somewhat of a paradox, as there appears to be a real syndrome (there is a clear pattern of convergent traits), yet, in this syndrome, the suite of convergent floral traits is present with very diverse pollinators and it is therefore unclear what drives the convergence. Johnson & Schiestl (2016) have pointed out that the problem with the syndrome of sapromyiophily is that it is used as an umbrella term for several oviposition site mimicry systems in which the pollinators are not necessarily functionally similar. The term makes specific reference to flies ("myio" is derived form the Greek "muia" meaning fly), yet the syndrome also includes species that are pollinated visited by both flies and beetles (Jürgens & Shuttleworth 2015). It may therefore be better to use functional definitions based on mimicry of different substrates, rather than a general classification of flower traits (Johnson & Schiestl 2016). There is great diversity in terms of floral morphology within the syndrome, where the idea of pollination syndromes was initially conceived to describe floral convergence to a specific pollinator or functionally similar set of pollinators (Johnson & Schiestl 2016). One of the challenges for understanding the evolutionary basis for the "sapromyiophilous syndrome" is that we have limited understanding of the particular pollinators and the functional significance of the unusual traits exhibited by the flowers. Even though possible functions have been suggested, there remains very little empirical evidence supporting these suggestions.

Sapromyiophily is often, but not exclusively, associated with carrion and faecal mimicry, which is believed to often represent a form of oviposition site mimicry or feeding sites (Jürgens & Shuttleworth 2015; Johnson & Schiestl 2016), although the evolutionary basis for this mimicry system is not always clear. Oviposition site mimicry is a strategy that some angiosperms utilise by imitating substrates for oviposition or larval development for gravid female insects (as well as males looking to mate) as well as potential food sources (such as carrion or dung) (Bolin et al. 2009; Chen et al. 2015; Johnson & Schiestl 2016; Jürgens et al. 2013). This strategy is deployed by a number of plant

genera around the world, to name a few: *Hydnora* (Hydnoraceae) (Bolin et al. 2009), *Amorphophallus* (Araceae) (Chen et al. 2015), *Helicodiceros* (Araceae) (Stensmyr et al. 2002), *Dracunculus* (Araceae) (Seymour & Schultze-Motel 1999), *Rafflesia* (Rafflesiaceae) (Beaman et al. 1988; Davis et al. 2007) as well as the stapeliads (Asclepiadoidae) (which includes the well-known genera *Stapelia*, *Orbea* and *Huernia*) (Bruyns 2005; Jürgens et al. 2006; Meve & Liede 1994; PŁachno et al. 2010). Even though a multitude of floral trait combinations can be associated with carrion and faecal mimicry (such as floral scent which will be discussed below), four traits commonly associated with carrion and faecal mimicry are: geoflory, trapping devices, thermogenesis and floral gigantism (Johnson & Schiestl 2016).

Geoflory (flowers situated at ground level) is particularly common in carrion mimics and therefore is likely to be an adaptation associated with oviposition site mimicry (Johnson & Schiestl 2016). The reason why plants exhibit geoflory is not yet understood, although it is likely to be due to the oviposition substrates or potential food sources (carrion or dung) being normally situated at ground level (Johnson & Schiestl 2016). Examples of sapromyiophilous plants exhibiting geoflory include *Hydnora,* which is a root holoparasite with a trapping chamber situated at ground level (Bolin et al. 2009), *Helicodiceros muscivorous,* where the spathe is often draped over the ground (Stensmyr et al. 2002) as well as the stapeliads where flowers are often positioned flat on the ground (Bruyns 2005; Johnson & Schiestl 2016; pers. obs.).

Trapping chambers are also strongly linked with carrion or faecal mimicry (Bröderbauer et al. 2013; Johnson & Schiestl 2016). Trapping chamber flowers usually lure insects into a chamber where exits are hindered, forcing the pollinator to make contact with reproductive parts of the flowers (Bröderbauer et al. 2013; Johnson & Schiestl 2016; Stensmyr et al. 2002). A good example of trapping chambers in the context of oviposition mimicry is *H. muscivorus* (the dead-horse arum). *Helicodiceros muscivorus* lures insects into a trap chamber, which surrounds the female florets (Stensmyr et al. 2002). The exit is then obstructed by spines and filaments, forcing the insect to fertilise the female florets if it is carrying pollen from another plant (Stensmyr et al. 2002). In the dead horse arum, these trapping chambers then stay closed until the female florets have become unreceptive, and the male florets at the entrance start to produce pollen. At this stage the spines and filaments wilt, allowing the insect to brush past the male florets (getting coated in pollen) and escape (Stensmyr et al. 2002). *Hydnora africana* also represents an interesting carrion mimic that uses trapping chambers (Bolin et al. 2009). This root holoparasite exhibits extremely reduced vegetative morphology where the actual chamber is situated below ground level (Bolin et al. 2009). This specific system makes use of glabrous walls in the androecial chamber to keep the insects detained. After

pollen is shed, insects escape as a result of textural changes to the androecial walls (Bolin et al. 2009). In contrast to the dead-horse arum, the chamber of *Hydnora africana* is made up of two parts; the androecial chamber and the gynoecial chamber, these two parts are joined by a ring of connate anthers with a central opening, which allows for pollinators to pass through and collect pollen (Bolin et al. 2009).

Thermogenesis is the ability of flowers to produce enough heat in order to raise their tissue temperature above ambient temperature (Barthlott et al. 2009; Johnson & Schiestl 2016; Seymour et al. 2009; Seymour et al. 2003). Thermogenesis is a strategy deployed by many ancient seed plants, including Araceae, Arecaceae, Aristolochiaceae, Annonaceae, Cycadaceae, Cyclanthaceae, Magnoliaceae, Nelumbonaceae and Nymphaeaceae (Seymour et al. 2009). Thermogenesis, along with scent production, is often situated in specialised organs called osmophores (Seymour et al. 2009; Vogel et al. 1990), although some plants contain two heat generating tissues with different functions (Seymour & Schultze-Motel 1999; Seymour et al. 2009). Seymour et al. (2009) showed that three species of *Hydnora* (*H. africana, H. abyssinica* and *H. esculenta*) are able to raise their temperature by up to 10 °C above ambient temperature. Another example is the well-known titan arum (*Amorphophallus titanum*) which is able to raise its temperature to 7 °C above ambient temperature (Barthlott et al. 2009), and interestingly, it has been shown that *H. muscivorus* exhibits maximum temperature elevations of 23.9 °C above ambient temperature (Seymour et al. 2003). Thermogenesis can serve multiple purposes for carrion and faecal mimics and these include: (1) increasing the volatilization of scent compounds (Johnson & Schiestl 2016; Seymour & Schultze-Motel 1999) (although for some carrion and faecal mimics (e.g. *Dracunculus vulgaris*) the heat production occurs outside the floral chamber and cannot increase volatilization of scent compounds) (Seymour & Schultze-Motel 1999), (2) an energy reward in the form of warmth (Johnson & Schiestl 2016; Seymour & Schultze-Motel 1999; Seymour et al. 2003), (3) resemblance to decaying carrion, as decaying carcasses exhibit increases in temperature for some days after death of the animal (Davis et al. 2008; Johnson & Schiestl 2016) and (4) thermogenesis can also assist with pollen germination and pollen tube growth (Johnson & Schiestl 2016; Seymour et al. 2009).

Floral gigantism is, interestingly, more prominent amongst carrion mimics than other forms of oviposition mimics (Johnson & Schiestl 2016). Floral gigantism, as the name implies, refers to plants with unusually large flowers. Some of these unusually large flowers that are associated with carrion and dung mimicry include the titan arum (*Amorphophallus titanum*) which can reach up to 3 m in height (Barthlott et al. 2009; Jürgens & Shuttleworth 2015), *Rafflesia arnoldii* which can reach up to 1 m in diameter and can weigh up to 7 kg (Davis et al. 2007)*,* the dead-horse arum (*Helicodiceros*

muscivorus) (Stensmyr et al. 2002) and *Stapelia gigantea* (Johnson & Jürgens 2010). There are a multitude of reasons why floral gigantism might be advantageous for flowers that mimic carrion, including: (1) for flowers that are visited by large bodied animals with high energy needs, the flowers need to store ample reward and protect of the reproductive parts of the flower (Davis et al. 2008), (2) visual preference of pollinators for larger a oviposition site such as a larger carcass (Davis et al. 2008; Johnson & Schiestl 2016), (3) increased heat production (Davis et al. 2008; Johnson & Schiestl 2016) and (4) a larger flower could result in greater amounts of scent production (Davis et al. 2008; du Plessis, Shuttleworth, Johnson unpubl; Johnson & Schiestl 2016; Valdivia & Niemeyer 2006). Greater scent emissions may increase fly attractions as studies have shown that blowfly attraction increases with increased emission of the volatile compound dimethyl trisulphide (Brodie et al. 2015; Johnson & Schiestl 2016). Further, Van der Niet et al. (2011) suggested that different flies are specialised to different sizes of carrion, therefore, floral gigantism could also represent a mechanism of specialising on a subset of available flies.

FLORAL SIGNALLING IN CARRION/FAECAL MIMICKING FLOWERS

Floral signalling is usually essential for attracting pollinators, and the signals that flowers exhibit can be either visual, olfactory or tactile. Extensive research has provided evidence that volatiles, which serve as an olfactory cue for visiting insects, have a strong link to the attraction of visitors in carrion mimicking flowers (Johnson & Jürgens 2010; Shuttleworth & Johnson 2010; Stensmyr et al. 2002). It can be assumed that olfactory cues would be the first step of attraction, as they likely serve as the long distance attractant before visual and tactile cues can play a role (Goodrich & Raguso 2009). Many, but not all, of the flowers that deploy this carrion or faecal mimicry strategy are known to emit oligosulphides (most notably dimethyl disulphide and dimethyl trisulphide), which are also emitted by decomposing carrion (Johnson & Jürgens 2010; Johnson & Schiestl 2016; Jürgens et al. 2013; Urru et al. 2011; Stensmyr et al. 2002). These oligosulphides are known to be very important to a number of carrion insects, although dimethyl trisulphide (DMTS) appears to be the most important as it induces antennal physiological responses as well as favourable behavioural responses (Brodie et al. 2014; Johnson & Schiestl 2016; Zito et al. 2014). Stensmyr et al. (2002) showed, in their study of the dead horse arum (*H. muscivorus*), the functional significance of floral volatiles (such as oligosulphides) for fly attraction. Dead horse arum florets only flower for two days, the first day of flowering is accompanied by foul odour production, whereas the second day is odourless (Stensmyr et al. 2002). The study found that there were significantly more fly visits during the first day in comparison to the second day. Stensmyr et al. (2002) then impregnated cotton rolls with scent blends

based on the scent profile (which consisted mostly of oligosulphides) of the dead horse arum and placed them inside the trapping chamber of the flowers during the second day of flowering. As a result, fly visits for both days were comparable (Stensmyr et al. 2002). Similar findings support the significance of oligosulphides for fly attraction in studies by Shuttleworth & Johnson (2010) as well as van der Niet et al. (2011), using plants from the *Eucomis* (Asparagaceae) and *Satyrium* (Orchidaceae) genera respectively. Zito et al. (2013) showed in their study on *Musca domestica* that these flies are also attracted by sweet odours (such as terpinolene, α-terpinene and linalool). Odour is clearly an important trait of these fly pollinated flowers, however, it likely functions in combination with other signals (visual or tactile) to improve the likelihood of successful pollination.

Visual signalling is likely to be effective as the short distance attractant that plays the most important role after the long distance attractant (olfactory cue) is successful (Jürgens et al. 2006). Visually, the flower not only represents a landing platform for flying insects such as carrion flies, but these flowers often also resemble animal carcasses in terms of colours, (browns, pinks and purples) (Johnson & Jürgens 2010). The addition of trichomes on the flowers could act as a visual cue in the resemblance of the skin to a mammal, where darkening of areas and patterning could also resemble open wounds or entry points into the carcass (Chen et al. 2015; Johnson & Schiestl 2016; Stensmyr et al. 2002). Although there are obviously convergent colours and patterns in carrion and faecal mimicking flowers, our understanding of the importance of these is limited. In terms of colour, Kugler's (1957) studies on the colour preferences of Sarcophagidae flies compared dark coloured models (such as brown and black) to light coloured models (such as yellow) in the presence of foetid scents as well as sweet scents. He found that in the presence of foetid scents, flies preferred dark coloured models and in the presence of sweet scents the preferred light coloured models. These early findings of Kugler (1957) were then reinforced by Brodie et al. (2014), who used inverted cups baited with dimethyl trisulphide as an attractant, and found that cups covered with reddish-brown cheesecloth were preferred to cups covered with white cheese cloth by gravid female blowflies. This was further reinforced by Aak & Knudsen (2011) using *Lucilia* and *Calliphora* flies as well as Wall & Fisher (2001) using *L. sericata*, where the preference for black models in the presence of carrion odours was evident. It should also be noted that contradictory results have been obtained by Moré et al. (2013), who tested the colour preference of flies for black versus and white models, in the presence and absence of a dimethyl disulphide and dimethyl trisulphide mixture. They found that more flies landed on the white models compared to black (Moré et al. 2013).

Studies have shown that blowflies make use of a range of sensilla on their antennae, labellum and ovipositor to assess the suitability of particular substrates for oviposition (Bänziger 1996; Johnson &

Schiestl 2016; Wallis 1962). This suggests that tactile cues may also be important for these insects. Tactile cues can be expected to be effective after long and short distance attraction (olfactory and visual cues) has been successful. A possible tactile cue found in some carrion flowers could be the presence of trichomes (for example some of the stapeliads) or roughly textured surfaces. A possible role of trichomes as a visual cue could be the resemblance to mammalian skin (Johnson & Jürgens 2010; Johnson & Schiestl 2016). Further, trichomes could also represent "flickering bodies", which are believed to attract flies through movement (Vogel 1954). Another possibility is that the subtle hints of movement may be reminiscent of a carcass filled with flies and therefore representing a suitable oviposition site. Trichomes have also been suggested to induce oviposition on flowers (Johnson & Schiestl 2016). Bänziger (2001), for example, argued that the hairs on *Rhizanthes infanticida* (Rafflesiaceae) stimulated oviposition in blowflies. Many carrion mimics are in fact glabrous and do not exhibit this tactile stimulation suggested by Bänziger (2001), although it should be noted that some temperate blowflies are known to lay eggs on glabrous substrates, such as rotting liver (Johnson & Schiestl 2016), and *Lucilia sericata* has been found to lay eggs on parts of rat carcasses where the hair is short (Charabidze et al. 2015). A major conceptual issue of this type of mimicry is that a too perfect imitation of an oviposition substrate cannot offer any evolutionary advantage to the flowers (Meve & Liede 1994). In other words, if this mimicry system becomes a preference as an oviposition site for the flies, this would lead to the extinction of the flies, and thus also the flowers that depend on them (Meve & Liede 1994).

STAPELIADS AS A MODEL FOR EXPLORING THE FUNCTIONAL SIGNIFICANCE OF TRAITS IN CARRION MIMICS

Stapeliads (Apocynaceae: Asclepiadoideae: Stapeliinae) are a monophyletic group of some 400 species of stem-succulent plants, many of which emit foul odours and exhibit unusual morphological and are believed to be pollinated by carrion associated flies. They are usually restricted to arid and semi-arid regions of the Paleotropics, ranging from southern Africa to East Africa, Arabia, southern Spain and Italy (Bruyns 2000; Bruyns 2005: Meve & Liede 1994). The floral diversity of stapeliads is not only notable between species, but also within species (see Bruyns 2005) (Figure 1). In terms of size, stapeliad flowers range from large flowers (such as *Stapelia gigantea)* of up to 400 mm in diameter to very small flowers (such as *Pseudolithos caput-viperae*) of 2.5 mm in diameter (Bruyns 2000). There is also remarkable variation in the shape of stapeliads, particularly in the shape of the corolla, which can range from almost flat to campanulate with cupular tube and lobes about the length of the tube (Bruyns 2000). Colouring of flowers can range from bright yellow (such as *Orbea lutea*)

to dark maroon (*S. hirsuta*, *S. leendertziae*) as well as more rarely green (such as the north African species *Orbea dummeri*) (Bruyns 2005; pers. obs.) (Figure 1). These colours are likely to form an interaction relationship with the scent emitted, as a bimodal cue complex has been described in which flies prefer different coloured models (or objects) in the presence of certain scent compounds (Brodie et al. 2014; Kugler 1957; Moré et al. 2013; Woodcock et al. 2014). The floral patterning found in stapeliads can also range from dark spots on lighter backgrounds, to lines in the corolla lobes (Bruyns 2005; Meve & Liede 1994; pers. obs.) (Figure 1). Trichomes are also often present on the larger flowered *Stapelia* species (such as *S. gigantea*, *S. gettliffei*, *S. grandiflora* and *S. hirsuta*), as well vibratile cilia along the margins of the corolla lobes which are found to move in just the slightest breeze (Bruyns 2005; Meve & Liede 1994) (Figure 1).

The pollination of these plants has not been studied in depth. Many stapeliads are considered to be pollinated by dung and carrion associated flies, with these fly assemblages seemingly made up of the fly families Calliphoridae, Sarcophagidae and Muscidae, although *Pachycymbium schweinfurthii is* reportedly visited by Drosophilidae flies (Meve & Liede 1994). Interestingly, pollination studies on this diverse group of plants (see Bruyns 2005, Figure 1) is extremely limited and most of the literature relies on few notes and anecdotal observations (Coombs 2010; Meve & Liede 1994). The levels of specialisation in terms of pollinators can vary greatly; according to Meve & Liede (1994) it would be possible for any fly to act as a pollinator, as long as the size relations between the fly proboscis and guide rail entrance is appropriate. An example of a very specialised stapeliad is *Orbea lutea* subsp. *lutea*, where this flower has been found to be pollinated by a single fly species in the genus *Antherigona* (Muscidae) (Shuttleworth et al. 2017). This study on *Orbea lutea* subsp. *lutea* showed how specific the size relations between the floral morphology and the pollinator can be, as the *Antherigona* flies were the only visitors small enough to be able to fit their heads between inner corolla lobes, which is necessary in order to make contact with the guide rails (Shuttleworth et al. 2017). Further, Coombs (2010) found that *S. hirsuta* var. *baylissi* is pollinated by Anthomyiidae flies, and suspects that it is likely by only one species. The cause of this specialisation to Anthomyiidae is unclear, as these flowers were also visited by Sarcophagidae, Muscidae, Calliphoridae, Luaxaniidae and Rhinophoridae and Tachinidae (Coombs 2010).
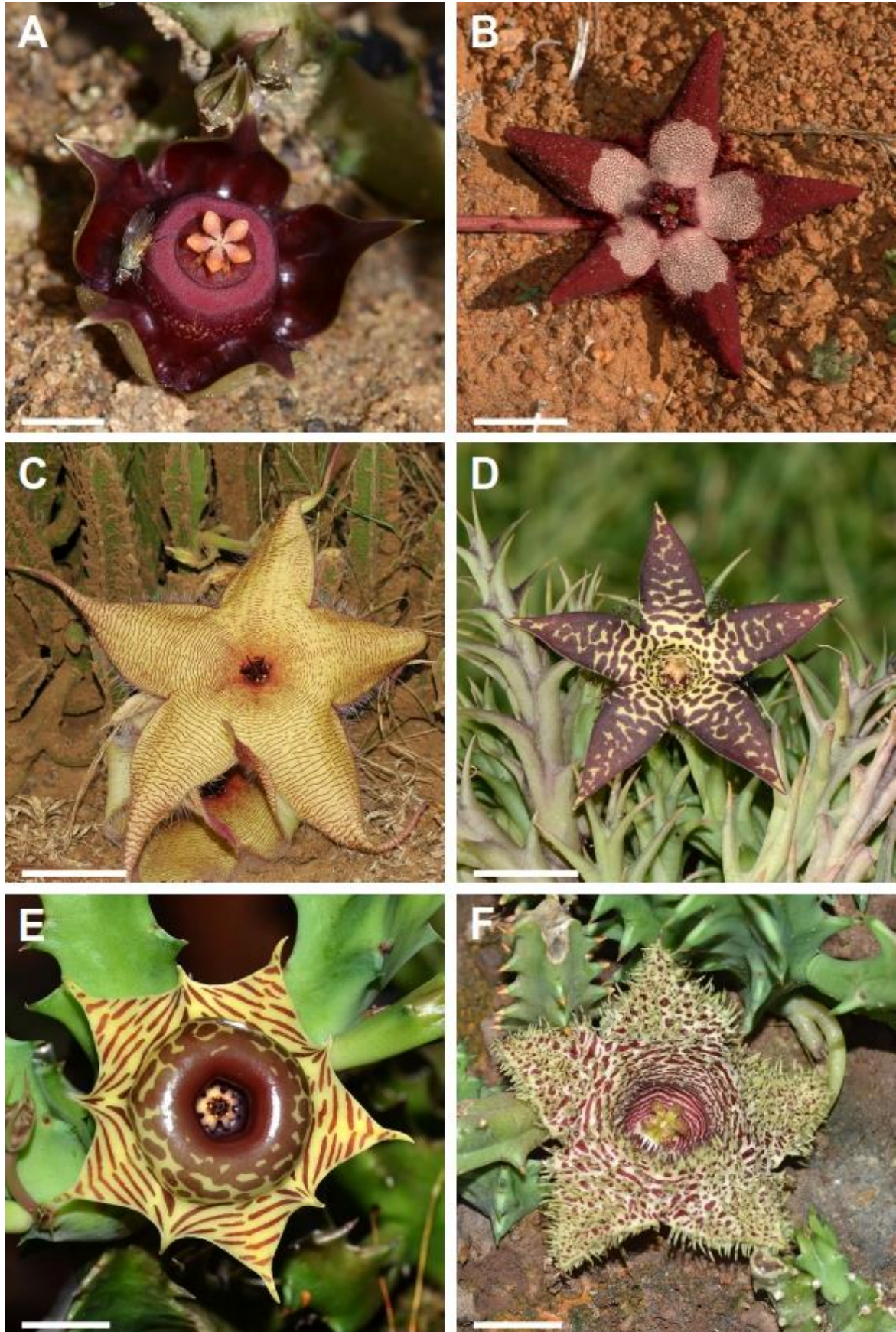
Figure 1. Diversity of stapeliad flowers. **A.** *Duvalia polita*. Scale bar = 1 cm. **B.** *Tromotriche pedunculata* subsp. *longipes*. Scale bar = 1.5 cm. **C.** *Stapelia gigantea*. Scale bar = 8 cm. **D.** *Orbea*

longidens. Scale bar = 3 cm. **E.** *Huernia zebrina*. Scale bar = 1 cm. **F.** *Huernia hystrix*. Scale bar = 1 cm. (All photos by A. Shuttleworth)

The pollination mechanism of stapeliads is a very specific process, since the removal of a pollinarium requires a certain amount of force and there appears to be a well-balanced size relationship between the pollinator and pollinarium (Meve & Liede 1994). The pollination of stapeliads, broadly, entails the removal of a pollinium (which attaches to the pollinator mechanically) followed by the insertion into another flower (Bruyns 2005). The exact mechanism of pollination can be divided into two processes (Bruyns 2005). For the removal of the pollinarium, the pollinator is required to make its way to the gynostegium of the flower. The potential pollinator is then expected to probe on the corona around the base of the guide rail, and a leg (rarely) or the proboscis may enter the base of the guide rail (Bruyns 2005). Once an appendage gets caught in between the guide rails, pulling up or outward will not release, only causing the appendage to run further up the rail (Bruyns 2005). The guide rail runs confluent with the slit of the corpuscle, resulting in the proboscis or leg getting caught in this slit (Bruyns 2005), if the alarmed fly is strong enough, it will be able to dislodge the pollinarium which is now attached to it (Bruyns 2005). For the insertion of a pollinarium, it is required that the insertion crest of the pollinarium gets caught in the guide rail, where any upward jerking movement would cause the insertion crest to slip in behind the rail (Bruyns 2005). Due to the insertion crest thickening above, and the guide rail becoming narrower, the pollinium would get stuck towards the top of the rail, and further pulling would break off the yoke (or the caudicle) between the pollinium and the rest of the pollinarium (which stays attached to the fly) (Bruyns 2005). This results in the pollinium being firmly lodged in between the guide rails, where pollen tubes grow to the stigma below the style head.

Many stapeliads exhibit foul odours reminiscent of carrion or faeces and floral volatiles are likely to play an important role in the attraction of pollinating flies. Jürgens et al. (2006) examined the volatiles produced by 15 species and showed that the compounds that make up these odours range from eight in *Pseudolithos cubiformis* to ninety-four in *Hoodia gordonii*. They also showed that the scent profiles were often dominated by only a few compounds (between one and six). However, it is still not clear how the combinations of these compounds influence the attraction of pollinators. Jürgens et al. (2006) also identified distinct chemotypes which they suggested represent mimicry of different substrates such as carrion (characterized by oligosulphide emission), faeces (characterized by emission of *p*-cresol, phenol and skatole) and urine (various acids). Interestingly, these flowers are believed to exhibit an odour gradient, where only some parts of the flowers emit odours (and some parts more than others) (Meve & Liede 1994). According to Meve & Liede (1994), the corolla lobes and the corona emit only slight odours, whereas the central parts emit stronger odours, and this is likely to guide pollinators to the central parts where the pollinia are situated. Volatiles commonly associated

with stapeliads include: acids, alcohols, aldehydes, aliphatic alkanes, benzenoids, ketones, monoterpenoids, nitrogen-containing compounds and sulphur-containing compounds (see Table 1). It is unlikely that all of these compounds are active compounds in the attraction of pollinators, however, oligosulphides have been shown be successful in the attraction flies (Shuttleworth & Johnson 2010; Stensmyr et al. 2002).

This study examined the pollination ecology and floral traits of two stapeliads, *Orbea variegata* and *Stapelia hirsuta* var. *hirsuta*. *Orbea variegata* flowers are glabrous and predominantly yellow, with dark red (sometimes black) patterning (blotching) on the corolla lobes (Bruyns 2005; pers. obs., see Figures 1 & 2, Chapter 2). In contrast, *S. hirsuta* flowers are covered in soft trichomes and are predominantly deep red to maroon in colour (Bruyns 2005; pers. obs., see Figure 1 & 2, Chapter 3). This allows for in depth studies of the functional significance of these opposing features (which are common features in carrion and dung mimicking plants). These two species both emit foul odours, which to the human nose resemble that of a decaying carcass, and are believed to be carrion mimics (Bruyns 2005). In addition, the level of specialisation of mimicry in terms of volatile emission (mimicry of carrion, carnivore faeces, dung and urine as suggested by Jürgens et al. (2006)) in stapeliads has not been explored in depth. For example, it was assumed that the dead horse arum mimics the scent of dead gulls, and it was shown that the scent profile of the model and the mimic were in fact very similar (Stensmyr et al. 2002). Some preliminary work has been done by Johnson & Jürgens (2010) comparing *O. variegata* (along with some other carrion and faecal mimics including other stapeliads) to certain models (dog and horse faeces, rat carcass and rotten meat), although it should be noted this work was done on cultivated *O. variegata* plants not wild populations. This study intends to uncover whether the odours of these two species are specialised mimics by mimicking just carrion or faces, or rather more general by mimicking the scents emitted by multiple models, and whether there are trade-offs associated with being generalist or specialist.

Table 1. Scent compounds identified from stapeliad odours which account for relative abundances of more than 10% of the total odour (from Jürgens et al. 2006; Johnson & Jürgens 2010). [a] = relative abundance > 25%, [b] = relative abundance > 50%.

Compound	Stapeliad species
Aliphatic Acids	
Butanoic acid	*Orbea semota* (N.E.Br) L.C. Leach.[a]
Aliphatic ketones	
3-Methyl-2-pentanone	*Stapelia asterias* Masson.
Alcohols	
2-Heptanol	*Hoodia currori* (Hook.) Decne.
Aldehydes	
Heptanal	*Huernia boleana* M.G. Gilbert.
	Piaranthus decorus (Masson) N.E.
	Stapelia asterias Masson.
Octanal	*Hoodia currori* (Hook.) Decne.
	Hoodia gordonii (Masson) Sweet.
	Huernia boleana M.G. Gilbert.[a]
	Huernia keniensis R.E. Fries.
	Piaranthus decorus (Masson) N.E.
	Pseudolithos cubiformis (P.R.O. Bally) P.R.O. Bally.
	Stapelia asterias Masson.[a]
Alkanes	
Tridecane	*Huernia hystrix* N.E.Br.
Pentadecane	*Huernia hystrix* N.E.Br.
Benzenoids	
p-Cresol	*Orbea semota* (N.E.Br) L.C. Leach.[a]
Benzoic acid	*Huernia keniensis* R.E. Fries.
	Piaranthus decorus (Masson) N.E.
Monoterpenoids	
α-Pinene	*Orbea verrucosa* (Masson) L.C.Leach.[a]
Sabinene	*Orbea verrucosa* (Masson) L.C.Leach.
(Z)-Ocimene	*Stapelia gigantea* N.E.Br.
(E)-Ocimene	*Stapelia gigantea* N.E.Br.[b]
Nitrogen-containing compounds	
2,5-Dimethylpyrazine	*Orbea variegata* (L.) Haw.
Indole	*Orbea variegata* (L.) Haw.[a]
Sulphur-containing compounds	
Dimethyl disulphide	*Huernia boleana* M.G. Gilbert.
	Huernia hystrix N.E.Br.[a]
	Huernia keniensis R.E. Fries.
	Orbea variegata (L.) L.C. Leach.
	Pseudolithos cubiformis (P.R.O. Bally) P.R.O. Bally.
	Stapelia gigantea N.E.Br.
	Stapelia leendertziae N.E.Br.[b]
Dimethyl trisulphide	*Hoodia gordonii* (Masson) Sweet.
	Huernia hystrix N.E.Br.[a]

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Orbea variegata (L.) L.C. Leach.[a]

Pseudolithos cubiformis (P.R.O. Bally) P.R.O. Bally.[b]

Stapelia leendertziae N.E.Br.[a]

Due to the foul odours that carrion and dung mimicking plants emit, this has often been the focus for studies on these plants and not many have explored the functional significance of other features of these plants. The functional significance of floral colours is one such feature. Colour preferences of carrion and dung associated flies have been studied as behavioural experiments in the context of the models that these flowers are mimicking (such as carrion or faeces) (Brodie et al. 2014; Kugler 1957), but has not yet been explored in the context of the flowers. Another floral feature often associated with carrion mimicking plants is the presence of floral trichomes (Johnson & Schiestl 2016). The function of trichomes has often been speculated on, but has not been tested in field based studies. Fly oviposition has also been linked with the presence of trichomes (Bänziger 2001), although the presence and magnitude of oviposition has not been established. Determining the functional significance of these unusual floral traits associated with carrion and dung mimicking flowers is of great importance, because if a trait influences the behaviour of a pollinator, it provides evidence that the specific trait evolved through selection by that pollinator. Therefore, these two species of stapeliads (*O. variegata* and *S. hirsuta* var. *hirsuta*) represent useful model systems with which to explore the functional significance of the unusual traits exhibited by carrion mimicking flowers.

The broad aims of this study were 1) to examine the pollination ecology of *O. variegata* and *S. hirsuta* var. *hirsuta* and 2) to explore the functional roles of some of the unusual traits exhibited by these two stapeliads for the attraction of fly pollinators. Investigations included detailed observations on the pollinators of flowers, and experimental invetsigations of the roles of colours and patterning in the attraction of flies by *O. variegata*, the role of trichomes on *S. hirsuta* var. *hirsuta* in the attraction of flies, effects of flower positioning on fly visits by *S. hirsuta* var. *hirsuta,* and the importance of visual versus olfactory cues in both *O. variegata* and *S. hirsuta*.

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CHAPTER 2

Connecting the dots and sniffing out functions: The importance of visual versus olfactory signals for the attraction of fly pollinators by *Orbea variegata*

ABSTRACT

Many flowers emit volatiles that are characteristic of the carrion and faecal substrates that are used as oviposition and feeding sites by saprophilous flies and beetles. The idea that these flowers are mimics of carrion and faeces is often further suggested by their visual and olfactory resemblance to these substrates. However, the relative importance of olfactory and visual signals for attraction of necro- and coprophagous insects to flowers remains unclear as most studies of the effects of scent and colour for attraction of these insects have been conducted in laboratory settings. To address these issues, I conducted a field-based study of the role of scent and colour in attraction of the pollinators of the succulent South African stapeliad *Orbea variegata.* These flowers attract flies belonging to the families Calliphoridae, Muscidae and Sarcophagidae, of which all three appear to be pollinators. *Orbea variegata* flowers have a yellow background colouring with black or dark red blotching. I found that the colours of these flowers are largely distinguishable from carrion, faeces and background rocks in a model of fly vision, although patterning on the lobes was indistinguishable from the dominant (yellow) colouring on the lobes. The foul odour of the flowers was dominated by dimethyl trisulphide, dimethyl trsulphide and phenol. Analysis suggested that the overall chemical composition of the scent was closer to that of faeces than of carrion, although the high relative abundance of dimethyl trisulphide in the samples suggests that *O. variegata* also mimics carrion. In the presence of the scent of *O. variegata* flowers flies were found to prefer black model flowers over yellow ones. However, model flowers with patterning resembling that on the corolla lobes of *O. variegata* were not preferred over models lacking such patterns. Flowers concealed by grass still received visits by flies, however these were significantly fewer than those received by visible flowers. This suggests that both scent and visual signals are important for attraction of flies in this system, although the patterning on the flowers does not appear to result from selection by fly pollinators. The functional significance of the

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unusual colour patterning of flowers of *O. variegata*, as well as the primary motivation of flies to visit these flowers, remain as unsolved puzzles.

INTRODUCTION

Carrion-mimicking flowers exhibit some of the most unusual and bizarre traits known in the angiosperms, but the evolution and functional significance of these weird features remain poorly understood. An important feature of carrion or faecal mimicry is the foul scent emission, and the composition of the volatiles emitted by these flowers has been found to be similar to the composition of the odours of carrion or dung (Johnson & Jürgens 2010; Jürgens & Shuttleworth 2015; Jürgens et al. 2006). The scent composition of putatively carrion or faecal mimicking flowers can often be quite diverse, although comparative case studies suggest that the scents emitted by these plants are often dominated by oligosulphides (dimethyl disulphide and dimethyl trisulphide), aliphatic acids or *p*-cresol (Kite & Hetterschieid 1997; Johnson & Jürgens 2010; Jürgens & Shuttleworth 2015; Jürgens et al. 2006; Jürgens et al. 2013; van der Niet et al. 2011). Interestingly, the scent composition of sapromyiophilous flowers (as well as fungi and mosses) is often convergent (Johnson & Jürgens 2010; Jürgens et al. 2013; Marino et al. 2009). Johnson & Jürgens (2010), for example, showed that a stinkhorn fungus (*Clathrus archeri*) also emits oligosulphides, phenol, indole and *p*-cresol. In addition to the convergence of floral scent of these carrion and faecal mimicking plants, colours and patterning are often convergent (Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015; Jürgens et al. 2006) suggesting that visual signalling may also influence the behaviour of pollinators. The functional significance of some of the floral volatiles as pollinator attractants has been confirmed in field studies (Shuttleworth & Johnson 2010; Stensmyr et al. 2002; van der Niet et al. 2011), but the interactions between scent and visual cues for the attraction of pollinators to carrion mimicking flowers have seldom been investigated outside of laboratory settings.

From an evolutionary perspective, the scent composition of different "sapromyiophilous" flowers has been suggested to mimic different substrates utilised by saprophilous insects, including carrion, faeces (carnivore, omnivore as well as herbivore), urine and decomposing plant tissue (Jürgens et al. 2006). Jürgens et al. (2006) suggested that characteristic compounds in the scents of carrion or faecal mimicking stapeliad flowers could be indicative of specific (different) models such as carrion, faeces

or urine. According to Jürgens et al. (2006), mimicry of herbivore dung is usually characterised by the presence of *p*-cresol, mimicry of carnivore dung and carrion is usually characterised by oligosulphides, heptanal and octanal and finally mimicry of urine is usually characterised by various acids. They argued that the chemical basis for these syndromes may be the presence of higher protein content in carrion as well as carnivore faeces which results in higher relative abundances of sulphur containing compounds (dimethyl disulphide and dimethyl trisulphide), whereas dung and decomposing plant material rarely contain these sulphur compounds (Jürgens & Shuttleworth 2015; Jürgens et al. 2006; Jürgens et al. 2013). It has been shown that carrion associated flies are attracted to flowers that emit sulphides (Moré et al. 2013; Shuttleworth & Johnson 2010; Stensmyr et al. 2002; van der Niet et al. 2011) and this is likely due to these sulphur containing compounds being indicators of protein rich food sources (Jürgens & Shuttleworth 2015; Jürgens et al. 2006). It is also worth noting that flowers may emit volatiles associated with multiple natural insect oviposition substrates and that the categories of mimicry are therefore not always discrete (Johnson & Schiestl 2016; Jürgens et al. 2006).

Floral colours and patterning in carrion or faecal mimicking plants are convergent across many different plant lineages (Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015; Jürgens et al. 2006), suggesting that they also play a major role in the attraction of flies and other carrion insects. Typical colouring includes deep reddish-brown or yellow, and many species exhibit deep-reddish or brown markings (either blotching or stripes) on a pale yellowish background (Beaman et al. 1988; Bruyns 2005; Barcelona et al. 2009; Johnson & Schiestl 2016; Lamprecht et al. 2002; van der Pijl 1961). Other colour-pattern combinations include yellow patterning on dark reddish backgrounds (such as *Stapelia hirsuta*) or in some cases completely yellow flowers (such as *Orbea lutea*) (Bruyns 2000; Bruyns 2005; Humeau et al. 2011; Shuttleworth et al. 2017). These specific colours and patterns have been suggested to contribute to the mimicry of carrion, where deep reds could imitate flesh (Bänziger 2001; Chen et al. 2015) and spots or lines could represent open wounds (Chen et al. 2015; Johnson & Schiestl 2016; Jürgens et al. 2006), although there is little empirical evidence to support this idea.

The diversity of floral colours found in stapeliads poses interesting questions about colour preferences of carrion and dung associated flies and the possible interaction effects between colours and odours. Kugler's (1957) preliminary studies on colour preferences of Sarcophagidae flies provided evidence that flies prefer dark coloured models in the presence of foul odours. Several studies have confirmed these findings with the use of oligosulphides (such as dimethyl disulphide and dimethyl trisulphide) in the presence of dark and light coloured models (Aak & Knudsen 2011; Brodie et al. 2014; Johnson

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& Schiestl 2016; Wall & Fisher 2001). However, opposing findings have also been reported where flies have been found to prefer light models (Moré et al. 2013).

Due to the foul odour being such a notable characteristic of carrion or faecal mimicry by flowers, much of the focus in previous studies has been on scent chemistry (Jürgens et al. 2013; Płachno et al. 2010; Vereecken & McNeil 2010). These olfactory cues have most likely evolved as a long distance attractant (Jürgens et al. 2006), whereas visual cues could be more important as a short distance attractant and draw attention to a possible landing site (Johnson & Jürgens 2010). This suggests that these signals may play complementary roles and the integration of these signals may thus be of great importance (Aak & Knudsen 2011). In other words, olfactory cues may be important to get the pollinators to the populations where these plants exist, whereas visual cues might assist the pollinator in selecting a target flower. This indicates that there is possibly a strong interaction between olfactory and visual cues exhibited by these flowers and that they go hand in hand to attract flies and manipulate their behaviour for pollination (Johnson & Schiestl 2016).

This study examined the relative importance of visual and olfactory signals in *Orbea variegata*, a well-known South African stapeliad with flowers which exhibit colour and odour traits that are typical of putatively carrion mimicking flowers. These include a strong carrion-like scent and pale yellow colouring with dark red (sometimes black) patterning (Figure 1). *Orbea variegata* was first formally described by Linnæus in his *Species Plantarum* (Linnæus 1753), and it was cultivated in Europe as early as the mid- to late-1600s (Bruyns 2005; Jürgens & Shuttleworth 2015). Its unusual flowers and succulent stems have appealed to enthusiasts and gardeners, and *O. variegata* is now widely cultivated around the world. Despite this popularity, there have been few studies of the species' ecology and the factors that may have influenced the evolution and pollination biology of its unusual flowers.

The broad aims of this chapter are to establish the pollinators of *O. variegata* and to explore the roles of visual versus olfactory signals in the attraction of pollinators. The specific aims were to (1) describe the pollination system and pollination success, (2) establish levels of natural fruit and seed set, (3) describe the floral scent and floral colours and compare these to putative models (carrion and dung), and (4) explore the importance of both olfactory and visual signals for the attraction of pollinators. I hypothesised that (1) flies prefer black coloured models in the presence of foul odours with a significant effect of patterning, and (2) visual and olfactory signals will have a synergistic effect on

the number of flies attracted.

METHODS AND MATERIALS

STUDY SPECIES AND STUDY SITE

Orbea variegata (L.) Haw is a widely distributed stapeliad in the south-western Cape with some of the northern populations occurring as far north as Lambert's Bay and Clanwilliam (Bruyns 2005). Some of the southern populations occur around Cape Town (more specifically Camps Bay, Clifton, the slopes of Signal Hill and Lion's Head as well as around the lower cable station on Table Mountain) and can be found along the Cape Peninsula and near the southern tip (Bruyns 2005). *Orbea variegata* prefers gentle, stony slopes where they are often fully exposed on rock slabs or under bushes (Bruyns 2005). Plant populations used in this study occurred in between the rocky outcrops behind the Clifton beaches in Cape Town (GPS: 33° 56 ' 38.082" S, 18 ° 22' 35.942" E, altitude: 37 m), as well as populations on the southern border of the Scarborough conservation village close to the Cape Peninsula (GPS: 34° 4' 31.997" S, 20° 33' 21.356" E, altitude: 122 m). This study was conducted during the flowering season of 2015, but with additional pollinator observations during the flowering season of 2016. At these sites, *O. variegata* produced buds around the end of March and flowered between April and May.

The flowers of *O. variegata* are typically pale yellow in colour with dark red (often almost black) blotching on the lobes and annulus (Figures 1 & 2). The variation in floral colour and patterning on these flowers is quite notable, particularly with respect to the size and colour of the blotching on the corolla lobes and the shade of yellow on the corolla lobes (Figure 2). Flowers are also variable in size. The flowers exhibit rotate ovate lobes which are reflexed with the centre of the corolla mostly flat and with an ascending ring-like annulus surrounding the gynostegium (Figure 1) (Bruyns 2005).

Figure 1. *Orbea variegata* plants and pollinators. **A.** Detail of pollinarium. Scale bar = 500 µm. **B.** Scanning electron microscope (SEM) image showing detail of corpuscle. Scale bar = 225 µm. **C, D** Whole plants in habitat at Scarborough showing a mature fruit with twin follicles and a single open flower respectively. Scale bar = 8 cm. **E.** Close-up of a single flower. Scale bar = 4 cm. **F.** *Musca domestica* fly visiting a flower. Note the inserted pollinium. Scale bar = 2 cm. **G.** Gynostegium with a removed pollinium and a pollinium that has not been removed. Scale bar = 0.5 cm. **H.** Close-up of the gynostegium. Scale bar = 2 cm. (p) pollinium, (ic) insertion crest, (c) caudicle, (wc) wing of corpuscle, (co) corpuscle, (a) annulus, (cl) corolla lobe, (g) gynostegium, (icl) inner corolla lobe, (dh) dorsal horn, (ocl) outer corolla lobe, (ip) inserted pollinium, (rp) removed pollinium and (nrp) pollinium that has not been removed.

Figure 2. Variation in floral patterning of *O. variegata* from Clifton and Scarborough.

FLORAL VISITORS AND THEIR BEHAVIOURS

Pollinators were observed at Clifton and Scarborough for approximately 60 mins per day over 12 days (commencing from 09h00 to 10h00). Where possible, representative visitors were collected and preserved for subsequent identification and assessment of pollen loads. In order to determine how extensively flies visit *O. variegata* flowers, visitation rates were determined by systematically recording all floral visitors over a time period of 120 mins per day for 12 days during the flowering season (commencing from 10h00 to 12h00). The mean visitation rate for each different visitor type was then calculated per hour. To determine how long the visitors stay on the flowers, visitors were observed for 60 mins and the time flies spent probing flowers was recorded. This was done over 12 days (commencing from 12h00 to 13h00).

Two aspects of fly behaviour were examined. Observing how the flies arrive on the flowers (landing directly on a flower, or landing on the plant and the walking onto a flower) was done to provide insight as to whether these flowers represent a strong visual target (or landing platform) or whether the short distance attraction could largely also be due to olfaction. Behaviour on the flowers (do the flies explore the gynostegium of the flower, or just the corolla lobes?) was observed to determine whether the flowers are successfully able to guide flies to the gynostegium, as flies often perch on the corolla. For both aspects, behavioural observations of all fly visitors lasted 60 mins at both field sites on separate days. The number of fly visitors exhibiting each specific behaviour was counted and scored, and the family recorded.

POLLINATION SUCCESS AND NATURAL FRUIT SET

Pollinium removal and insertion rates were scored for *O. variegata* at both field sites. Forty-nine and 121 open flowers (of varying ages) were inspected at Clifton and Scarborough respectively. Since the pollinia of *O. variegata* are relatively large, the insertion or removal of a pollinium can be seen with the naked eye. The pollen transfer efficiency (PTE) was then calculated as the proportion of removed pollinia that are inserted.

To determine the success of *O. variegata* in terms of natural fruit and seed set at Scarborough, buds and flowers of four clones of different sizes were counted early during the 2015 flowering season to determine the number of flowers per clone. All additional flowers developing on these selected clones were scored (until the end of flowering season). The fruits of each selected clone were later collected, and the fruits and seeds counted. *Orbea variegata* flowers generally produce two follicles per fruit (although in some cases only one) (see Figure 1). The percentage of flowers developing fruit, average seed set per follicle as well as the mean seed set per fruit were calculated.

FLORAL SCENT ANALYSIS AND COMPARISON WITH VOLATILES PRODUCED BY POTENTIAL MODELS

Floral volatiles were collected using dynamic headspace sampling methods. For this, Nalophan polyacetate bags (Kalle, Germany) were used to enclose the flowers and volatiles were sampled from the bags using dynamic headspace extraction methods. A total of five and seven flowers were sampled

(one flower per sample at about mid-day) from Clifton and Scarborough respectively. Air was pumped (using a PAS500 Personal Air Sampler from Spectrex, Redwood City California) from the polyacetate bag through small volatile traps filled with 1 mg of Tenax® TA (60/80) (Supelco™; Bellefonte, PA, USA) and 1 mg of Carbotrap® B (20–40 mesh) (Sigma-Aldrich Co.; St Louis, MO, USA) for 90 mins per sample, using a flow rate of 150 ml/min. Control samples were taken, and consisted of only ambient air in the polyacetate bag being pumped through a volatile trap. Headspace samples were then analysed by coupled gas chromatography and mass spectrometry (GC-MS), on a Varian (Palo Alto, CA) CP3800 gas chromatograph fitted with a Varian 1079 injector with a ChromatoProbe thermal desorption device. A Bruker BRSwax column was used, and a known amount of methyl benzoate injected under identical conditions to samples was used as a standard to calculate emission rates. The capillary column was 30 m long with a 0.25 mm internal diameter and a 0.25 μm film thickness. Helium was used as the carrier gas with a flow rate of 1 ml/min. The injector was held at a temperature of 40 °C for 2 min with a 20:1 split, followed by a temperature increase to 200 °C (over a minute) in splitless mode for thermal desorption. The GC oven temperature was maintained at 40 °C for 3 min after which it was raised to 240 °C (at 10 °C per min) and maintained for 12 min. A Varian 1200 quadrupole mass spectrometer was used (coupled with the GC) in electron-impact ionization mode at 70eV with the detector voltage set by Extended Dynamic Range (EDR). The separated compounds were then cross referenced against a library of known compounds (NIST2011, version 2.0) using the Varian Workstation software (version 6.8). Retention times and mass spectra of authentic standards and published relative retention times (Kovats indices) were used to confirm library identifications wherever possible. A Similarity-Percentages (SIMPER) analysis was conducted in Primer 6 to identify the compounds that best characterise the scent bouquet of *O. variegata.* This analysis calculates the percentage contributions of each compound to average overall Bray-Curtis similarity between samples within a group (Clarke & Gorley 2006).

To explore the similarity between the odour of *O. variegata* and assumed models, we compared the odour to that of carrion, carnivore faeces and dung using non-metric multidimensional scaling (NMDS) based on relative amounts (i.e. the percentage of the headspace). This analysis was based on Bray-Curtis similarities and data were first square root transformed to downweight the influence of dominant compounds (Clarke & Gorley 2006). This was implemented in Primer 6 (scent data for carrion, carnivore faeces and dung were obtained from A. Shuttleworth & A. Jürgens unpublished data; see Table 1 for details). In addition, the scent profiles of 26 non-carrion mimicking asclepiad (Apocynaceae: Asclepiadoideae) species were included in the NMDS analysis to represent an outgroup to assess advergence to the odours of potential models (see Table 2) (scent data for the 26 non-carrion mimicking species were obtained from Shuttleworth & Johnson 2012 and references

therein). A one-way Analysis of Similarity (ANOSIM; see Clarke & Gorley 2006) was conducted in Primer 6 to test for differences between the scent profile of *O. variegata* flowers and each of the 26 asclepiads (Table 2), as well as the carrion, carnivore faeces and dung odours. ANOSIM generates a test statistic (R) resulting from 10,000 permutations of the sample labels such that values close to 1 indicate low similarity, and values close to 0 indicate high similarity (Clarke & Gorley 2006). This non-parametric permutation procedure is based on the similarity matrix underlying the ordination (Clarke & Gorley 2006).

Table 1. Animals used for the scent analysis of of three-day old carrion, carnivore faeces and dung (Kheswa, N., Shuttleworth, A. & Jürgens, A. unpublished data)

Carrion scent samples	Carnivore faeces scent samples	Dung scent samples
Species (Common name)	**Species (Common name)**	**Species (Common name)**
Dispholidus typus Smith. (Boomslang)	*Panthera tigris* Linnaeus. (Tiger)	*Struthio camelus* Linnaeus (Ostrich)
Cossypha caffra Linnaeus. (Cape robin)	*Panthera leo* Linnaeus (Lion)	*Tapirus* sp. Brünnich. (Tapir)
Rattus norvegicus Berkenhout. (Lab rat)	*Panthera pardus* Linnaeus. (Leopard)	*Ceratotherium simum* Burchell. (White rhino)
Bitis arietans Merrem. (Puff adder)	*Cryptoprocta ferox* Bennett. (Fossa)	*Equus quagga* Boddaert. (Zebra)
Rhabdomys pumilio Sparrman (Three-striped mouse)		*Lama glama* Linnaeus (Llama)
Tilapia sp. Smith.		*Tragelaphus angasii* Angas. (Nyala)
Amietophrynus gutturalis Power. (Toad)		*Macaca* sp. Lacépède. (Java monkey)
Amblyospiza albifrons Vigors. (Thick billed weaver)		*Sus scrofa domesticus* Erxleben. (Pot belly pig)
		Dromaius novaehollandiae Latham. (Emu)
		Pan troglodytes Blumenbach. (Chimpanzee)
		Loxodonta africana Blumenbach. (Elephant)
		Syncerus caffer Sparrman. (Buffalo)
		Camelus sp. Linnaeus. (Camel)
		Papio ursinus Kerr. (Chacma baboon)

Table 2. List of asclepiad species (Apocynaceae: Asclepiadoideae) used for NMDS as well as their main pollinators (Shuttleworth & Johnson 2012).

Asclepiad species	Pollinator
Asclepias albens Schltr.	Chafer
Asclepias crispa Schltr.	Generalist
Asclepias cucullata Schltr.	Bee
Asclepias dregeana Schltr.	Bee
Asclepias gibba Schltr.	Bee
Asclepias macropus Schltr.	Wasp
Asclepias woodii Schltr.	Chafer
Miraglossum pilosum (Schltr.) Kupicha.	Wasp
Miraglossum verticillare (Schltr.) Kupicha.	Wasp
Pachycarpus acidostelma M.Glen & Nicholas.	Chafer
Pachycarpus appendiculatus E.Mey.	Wasp
Pachycarpus asperifolius Meisn.	Wasp
Pachycarpus campanulatus N.E.Br.	Wasp
Pachycarpus concolor E.Mey.	Chafer
Pachycarpus coronarius E.Mey.	Unknown
Pachycarpus grandiflorus E.Mey.	Wasp
Pachycarpus natalensis N.E.Br.	Wasp
Pachycarpus plicatus N.E.Br.	Chafer
Pachycarpus scaber N.E.Br.	Chafer
Periglossum angustifolium Decne.	Wasp
Xysmalobium gerrardii Scott Elliot.	Generalist
Xysmalobium involucratum Decne.	Chafer
Xysmalobium orbiculare D.Dietr	Wasp
Xysmalobium parviflorum Harv. ex Scott Elliot	Fly
Xysmalobium stockenstromense Scott Elliot.	Wasp
Xysmalobium undulatum (L.) W.T.Aiton	Wasp and chafer

NECTAR

Orbea variegata flowers were inspected for the presence of nectar. This was done early in the mornings (c. 09h00) before flowers were extensively visited by pollinators. A total of 20 flowers were inspected at Scarborough, and a total of 10 flowers at Clifton.

ANALYSIS OF FLORAL COLOURS AND THE COLOURS OF POTENTIAL MODELS

The spectral reflectance of the flowers between 300 and 700 nm was determined using reflectance spectroscopy. An Ocean Optics S2000 spectrometer, with a QR-200-7-UV-vis fibre optic retention probe (200 – 850 mn; 200 μm) held at 45° to the surface of the flower. The light source used was an Ocean Optics DT-Mini (deuterium tungsten halogen) with a spectral range of approximately 200nm – 1100nm. The colour spectra were measured for four different coloured parts of *O. variegata*: yellow on corolla lobes, the black/dark red blotches on corolla lobes, yellow on outer corona and black blotches on outer corona. Six separate flowers (from Scarborough) were used, and each data point was taken as an average of three spectral measurements.

In order to determine how these floral colours are perceived by flies, the reflectance spectra were plotted as loci in the fly colour vision model suggested by Troje (1993). This model is based on receptor sensitivities of *Musca domestica* flies published by Hardie & Kirschfeld (1983) and behavioural experiments with *Lucilia* sp. flies by Troje (1993). The results of these experiments suggested that flies exhibit a categorical colour vision system in which there is no discrimination between colour spectra unless they fall in discrete categories (Troje 1993; Arnold et al. 2009). The Troje (1993) model assumes two antagonistic colour opponent subsystems: R 7p/R 8p and R 7y/R 8y. The colour categories are formed by evaluating whether the excitations between p and the y receptors are positive or negative, giving rise to four possible categories; p+ y+, p+ y-, p- y+ and p-y- (Troje 1993; Arnold et al. 2009). This model does not take into account achromatic colour contrasts which could also play a role in fly vision when it comes to visual cues in floral pollination (Shuttleworth et al. 2017). It was assumed that fly receptors would be adapted to a green leaf background spectrum and a mean spectrum of green leaves from typical Fynbos habitat close to the sites (published in Welsford et al. 2016) was used for the background (adaptation) spectrum in the model. The receptor excitations were calculated using methods described by Chittka (1996).

To compare the spectra of *O. variegata* flowers to the colours of potential models, I also measured the spectral reflectance of dung and rotting ox liver using the methods described above. Horse and sheep dung was collected from the Onderstepoort (veterinary) campus at the University of Pretoria, and liver (obtained from a supermarket) was left out to decompose for three days before spectral reflectance was measured. All measurements were taken on 27 July 2015, and dung was obtained on 26 July 2015. The colour spectra were interpreted using the fly colour vision model described above (Troje 1993).

EXPERIMENTS TESTING THE FUNCTIONAL SIGNIFICANCE OF VISUAL AND OLFACTORY SIGNALS

To explore the relative importance of olfactory versus visual signals, I conducted an experiment in which flies were offered a choice between concealed and exposed flowers. This experiment was carried out at Scarborough between 11h00 and 14h00 on sunny days between late April and late May 2015. Two fresh *O. variegata* flowers were placed a meter apart and one was covered with dried grass so it was not visible. A pile of grass was placed approximately a meter from both flowers (to form a triangle) as a negative control. Fly visits to any of the three points were then recorded over a 90 min period, and this was repeated five times on separate days. Only visits (physical contact) to the flower or grass piles were scored. The concealed flower and visible flower were switched every 15 min as a control measure. Contamination with *O. variegata* floral volatiles was avoided by ensuring that the negative control grass pile never made physical contact with a flower.

Fly colour preferences generally have been tested for light versus dark coloured models (Brodie et al. 2014; Kugler 1957; Woodcock et al. 2014), and it has been suggested that in the presence of foetid odours, flies prefer darker models. To explore the importance of floral colour in *O. variegata* flowers, I conducted experiments using yellow and black cardboard model flowers. This experiment was aimed at determining which colours are preferred by flies in the presence of *O. variegata* scent, and was carried out at Scarborough after 13h00 between late April and late May 2015. In this experiment four flowers were constructed from cardboard (according to the dimensions of *O. variegata*), two black and two yellow. The spectral reflectance of the black and yellow cardboard was measured using the methods described above to confirm that these were similar to reflectance of reddish-black markings and yellow of *O. variegata* flowers across the range of wavelengths to which fly eyes are sensitive (300-600 nm) (Hardie & Kirschfeld 1983). One black flower and one yellow flower was placed over fresh *O. variegata* flowers (to provide scent) and the remaining black flower and yellow flower were left unscented. These flowers were then placed in the field along with an unmanipulated flower as a control, approximately one meter apart (in a circular formation) and fly visits to these flowers were recorded over a period of 90 min. Cardboard flowers covering real flowers were switched every 15 min, while unscented cardboard flowers were left untouched (to control for any possibility that the cardboard may retain scent from real flowers). Positions of flowers were also changed (to control for learning by flies) every 15 mins. Two types of yellow cardboard were used for this experiment as stationary stores ran out of stock of the initial yellow cardboard used (this was included as a factor in the statistical model). This was repeated five times on separate days (with fresh flowers with every replicate) for each yellow cardboard type (but using the same black cardboard

throughout). Fly visits (separated into families) were once again only scored if contact (landing on or walking onto flower) was made by the visitor.

To explore the role of the blotching in the attraction of flies, I conducted choice experiments using model cardboard flowers. These experiments were carried out at Scarborough. Flowers were designed and cut from cardboard based on the shape and size of *O. variegata* flowers in the following designs: plain yellow cardboard, yellow cardboard with small black blotches (1 mm in diameter), yellow cardboard with medium black blotches (2 mm in diameter) and yellow cardboard with large black blotches (4 mm in diameter) which were drawn on using a black marker. These cardboard flowers were then placed over real flowers to provide the scent bouquet associated with *O. variegata*, and a real flower without any cardboard was used as a control. The flowers were placed in the field with approximately 1 meter spacing between flowers. To control for the possible effects of volatiles emanating from the ink used to make black markings, additional black markings were made on the underside (concealed from view) of all cardboard flowers and real flowers used. Cardboard flowers and real flowers were switched and positions of designs were changed every 15 minutes. The flowers were observed for 90 min. This was repeated a total of ten times on separate days with new sets of flowers each time. These experiments were once again repeated with two different types of yellow cardboard. Fly visits (separated into fly families) were counted, and a visit was considered as the act of a fly making contact with a model flower.

STATISTICAL ANALYSES

Data were analysed using generalized estimating equations implemented in SPSS 23 (IBM Corp). Models that analysed proportions incorporated a binomial error distribution and logit link function, while those that analysed counts incorporated a Poisson distribution and log link function or linear distribution with an identity link function. Significance was tested using chi-square statistics (Wald and Generalised score) and the Sequential Sidak procedure was used for post-hoc tests. Means and standard errors presented in graphs were obtained by back-transformation from the logit or log scales.

RESULTS

FLORAL VISITORS AND THEIR BEHAVIOURS

In terms of visitor assemblage, the fly families observed on and around the flowers included Calliphoridae, Muscidae and Sarcophagidae (Table 3). A total of 53 flies were collected, of which most were Muscidae and Calliphoridae. The pollen loads of these respective fly families were lowest among Calliphoridae, and highest for Sarcophagidae. Muscidae flies were the most common visitors with Calliphoridae being less active. Sarcophagidae were never observed visiting flowers, although many individuals caught near the flowers were carrying pollinia. Flies were often seen arriving on the corolla lobes and then making their way to the gynostegium. To remove pollinia, the flies had to face the guide rails while probing a small cavity at below the guide rails at the base of the gynostegium. While doing so, the labium was sometimes trapped between the guide rails and picked up a pollinium as the fly withdrew (Figure 1). In all observed cases, the individual struggled to free itself before flying off.

Table 3. Insect visitors collected on *O. variegata* flowers and plants, indicating the number with found to carry pollinia at both field sites. [a] = fly collection was lost before species-level identifications could take place. [b] = flies identified from photographs. All pollinia were placed on fly mouthparts. Sarcophagidae were never observed visiting flowers, but were collected in close proximity to the flowers.

Species	No. observed Scarborough	No. observed Clifton	No. carrying pollinia	Flowering season
Calliphoridae				
Calliphoridae sp. 1[a]	14(1)	4	1	2015
Lucilia cuprina Wiedemann.	2	0	0	2016
Lucilia sericata[b] Meigen.	3	1	0	2015
Lucilia sericata Meigen.	3	0	0	2016
Unidentified Calliphoridae	81(1)	1	1	2015
Muscidae				
Muscidae sp. 1[a]	20(4)	4	4	2015
Musca domestica[b] Linnaeus.	4	4	0	2015
Musca domestica Linnaeus.	5	0	0	2016
Unidentified Muscidae	206(6)	25	6	2015
Sarcopagidae				
Sarcophagidae sp. 1[a]	6(5)	0	5	2015

The visitation rates of Muscidae and Calliphoridae (Table 4) were found to differ significantly ($\chi^2 = 57.522$, $P < 0.001$. Again, a significant effect of fly family was found in the amount of time spent probing flowers (Table 4) ($\chi^2 = 9.078$, $P = 0.003$,).

Table 4 Mean fly visits to *O. variegata* flowers per hour and the mean time spent probing on flowers.

	Muscidae	Calliphoridae
Fly visits per hour	2.3 ± 0.1 (n = 231)	0.9 ± 0.1 (n = 86)
Fly time per flower (mins)	2.3 ± 0.1 (n = 231)	1.8 ± 0.1 (n = 82)

No difference was found between fly families in terms of the percentage of flies exploring the gynostegium of *O. variegata* ($\chi^2 = 0.010$, $P = 0.922$). In terms of the percentages of flies landing directly on flowers, no significant difference was found between Muscidae and Calliphoridae ($\chi^2 = 2.792$, $P = 0.095$).

Table 5. Percentage of fly visitors that explore the gynostegium of *O. variegata* flowers and the percentage of flies that land on flowers.

	Calliphoridae	Muscidae
Percentage of flies that probed the gynostegium	92.7 ± 2.8 (n = 231)	91.8 ± 2.0 (n = 82)
Percentage of landings on flowers	73.2 ± 3.6 (n = 231)	65.4 ± 3.2 (n = 82)

POLLINATION SUCCESS AND NATURAL FRUIT SET

Pollen transfer efficiency (PTE) was 4.5% at Clifton, and 9.5% at Scarborough. At Clifton, a mean of 4.2 ± 0.5 pollinia were removed per flower, and a mean of 0.2 ± 0.1 were inserted (n = 49 flowers), and at Scarborough, a mean of 4.6 ± 0.3 pollinia were removed and a mean of 0.5 ± 0.1 were inserted (n = 121 flowers).

The *O. variegata* clones observed developed a total of 116 flowers during the 2015 flowering season. The number of fruits collected after flowering season totalled to 63 fruits, with 7 flowers producing a single follicle. The number of *O. variegata* flowers that produced fruit and were successfully pollinated equalled to 63 flowers (37.95%). The mean number of seeds per follicle were 169.8 ± 1.1 and the mean no seeds per fruit were 316.9 ± 5.7 seeds per fruit.

FLORAL SCENT ANALYSIS AND ASSESSMENT OF THE VOLATILES PRODUCED BY POTENTIAL MODELS

The odour of *O. variegata* flowers was dominated by phenol, dimethyl disulphide and dimethyl trisulphide, although several other compounds including isobutyl isovalerate, anisole and indole often also contributed high relative amounts in some samples (Table 6). The total number of compounds found per sample was low, ranging from 15 compounds to 22. The total volatiles (ng) emitted per flower per hour was also relatively low across all samples, with the highest being 130.9 ng/flower/hour and the lowest being 19.5 ng/flower/hour (Table 6). The SIMPER analysis identified phenol, dimethyl disulphide and dimethyl trisulphide as compounds which particularly characterise the scent of these flowers, with these three compounds accounting for over 70% of the contribution to average similarity between samples (Table 7).

Table 6. Relative amounts (%, based on peak area) of volatiles identified from headspace samples of *O. variegata* at field sites; Clifton and Scarborough (Western Cape, South Africa). tr = compounds that represent < 0.005% of total odour blend. Identification criteria: A = library match only, B = library match and confirmation with published retention index and C= library match and confirmation with synthetic standard. [a] = found in controls, but in significantly smaller amounts than in samples.

Compound	Kovats	ID criteria	Clifton					Scarborough						
			1	2	3	4	5	1	2	3	4	5	6	7
Aliphatic compounds														
Alcohols														
Heptan-2-ol	1307	A	3.4	0.4	2.4	0.8	0.6	tr	1.6	0.9	-	0.8	-	0.2
4-Methylhexan-1-ol	1403	A	0.1	0.5	0.3	2.5	5.1	-	0.7	0.5	0.3	-	0.1	0.1
Oct-1-en-3-ol	1425	C	0.7	0.9	0.2	3.3	1.8	7.9	3.1	4.0	1.4	0.5	1.0	0.8
Ketones														
Heptan-2-one	1219	A	3.7	0.5	4.1	1.4	0.8	-	0.6	-	-	3.5	-	-
Nonan-2-one	1376	A	-	-	-	-	-	1.2	-	tr	tr	-	-	tr
Esters														
Isopropyl isovalerate	1168	A	-	-	21.7	-	22.1	-	43.3	44.0	6.3	39.4	0.4	tr
Isobutyl isovalerate	1216	A	-	-	-	4.6	8.0	-	1.8	tr	2.6	tr	6.9	1.6
Isopropyl tiglate	1251	A	3.6	0.4	6.0	1.8	0.9	tr	1.5	9.7	2.4	3.2	0.1	0.2
Hexyl acetate	1279	C	-	-	-	0.4	0.1	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl acetate	1312	C	-	-	-	-	-	5.9	-	tr	-	tr	tr	-
Aromatic compunds														
Anisole	1334	C	-	3.5	2.5	2.0	8.2	11.1	1.6	4.9	8.7	23.4	1.5	4.2
Phenylpropan-2-one	1738	A	2.1	tr	0.1	0.2	tr	10.4	tr	-	-	-	0.1	0.1
1-Phenyl-2-propanol	1850	A	3.6	tr	0.3	0.2	tr	7.4	tr	-	tr	-	0.1	tr
Phenylethyl alcohol	1945	C	1.0	0.1	-	0.8	0.4	0.7	0.3	0.1	0.1	tr	1.6	0.4
Phenol	2023	C	20.9	15.2	6.2	22.9	25.1	13.3	16.7	28.0	27.0	15.1	48.8	23.8
Phenylethyl isovalerate	2040	A	-	-	-	-	-	-	tr	tr	tr	-	1.1	0.3
3-Phenylpropanol	2086	A	tr	-	-	-	tr	tr	tr	tr	0.5	tr	-	0.2
p-Cresol	2107	C	1.3	tr	tr	0.1	tr	0.4	tr	tr	tr	tr	0.1	0.1

Nitrogen-containing compounds

Trimethylamine	600	A	-	tr	-	-	-	-	tr	tr	-	-	tr	-
Indole	2486	C	tr	1.0	tr	2.9	0.3	36.1	10.0	0.6	1.0	0.1	24.8	3.4
Sulfur-containing compounds														
Dimethyl disulfide	1070	C	24.5	51.2	24.1	38.8	19.6	tr	8.9	2.8	29.8	8.3	4.7	15.2
Dimethyl trisulfide	1362	C	29.9	26.3	30.6	14.9	4.4	tr	9.2	3.8	19.7	5.4	6.8	48.8
Dimethyl sulfone	1941	C	3.4	0.1	1.3	1.0	0.5	2.3	0.2	0.5	0.1	0.2	0.2	0.2
Unknown compounds														
m/z: 114*,55,83,41,81,70,67	1446		-	-	-	0.9	1.6	-	-	-	-	-	-	-
m/z: 178*,94,57,85,105,41,77	1820		-	tr	-	0.2	tr	-	0.1	tr	tr	tr	1.1	0.4
m/z: 114*,55,42,56,41,84,39	1912		1.7	0.1	-	0.6	0.4	3.3	0.3	0.2	0.3	0.1	0.5	0.2
Aliphatics			11.6	2.6	34.8	14.7	39.5	15.0	52.5	59.1	12.9	47.4	8.5	2.9
Aromatics			28.8	18.7	9.2	26.1	33.6	43.3	18.6	33.0	36.2	38.5	53.4	28.9
Nitrogen-containing compounds			0.0	1.0	tr	2.9	0.3	36.1	10.0	0.6	1.0	0.1	24.8	3.4
Sulfur-containing compounds			57.9	77.6	56.1	54.7	24.6	2.3	18.4	7.1	49.6	13.9	11.8	64.1
Unknowns			1.7	0.1	-	1.7	2.0	3.3	0.4	0.2	0.3	0.1	1.6	0.6
Number of compounds			16	18	15	20	22	15	22	21	19	18	20	21
Total emission (ng/flower/hour)			48.7	124.9	77.5	82.4	96.1	45.5	42.9	16.1	38.2	19.5	130.9	67.6

Table 7. Compounds characterizing the odour of *O. variegata* based on a SIMPER analysis. Contribution (%) represents the contribution to overall Bray-Curtis similarity within samples of *O. variegata*. Sim/SD = % contribution divided by standard deviation. High percentage contributions and high sim/SD values would indicate compounds that best characterize the species' scent bouquet.

Compound	Sim/SD	Contribution (%)
Phenol	2.66	34.10
Dimethyl disulphide	1.08	20.96
Dimethyl trisulphide	1.01	17.44
Isoprotyl isovalerate	0.45	9.88
Anisole	1.12	5.67
Indole	0.37	2.72

The NMDS comparing scent profiles of *O. variegata* to putative models (carrion, carnivore faeces and dung) and other asclepiads (see Table 2), places *O. variegata* and the asclepiads on either side of the dung, faeces and carrion (Figure 3). This suggests that the scent composition of *O. variegata* has more similarities with carrion, faeces and dung than with the other asclepiads. A pairwise ANOSIM analysis showed that the scent composition of *O. variegata* is the most similar to the scent composition of dung (R = 0.695) with the least similarity to the asclepiads (range of R = 0.973 – 0.988) (Table 6).
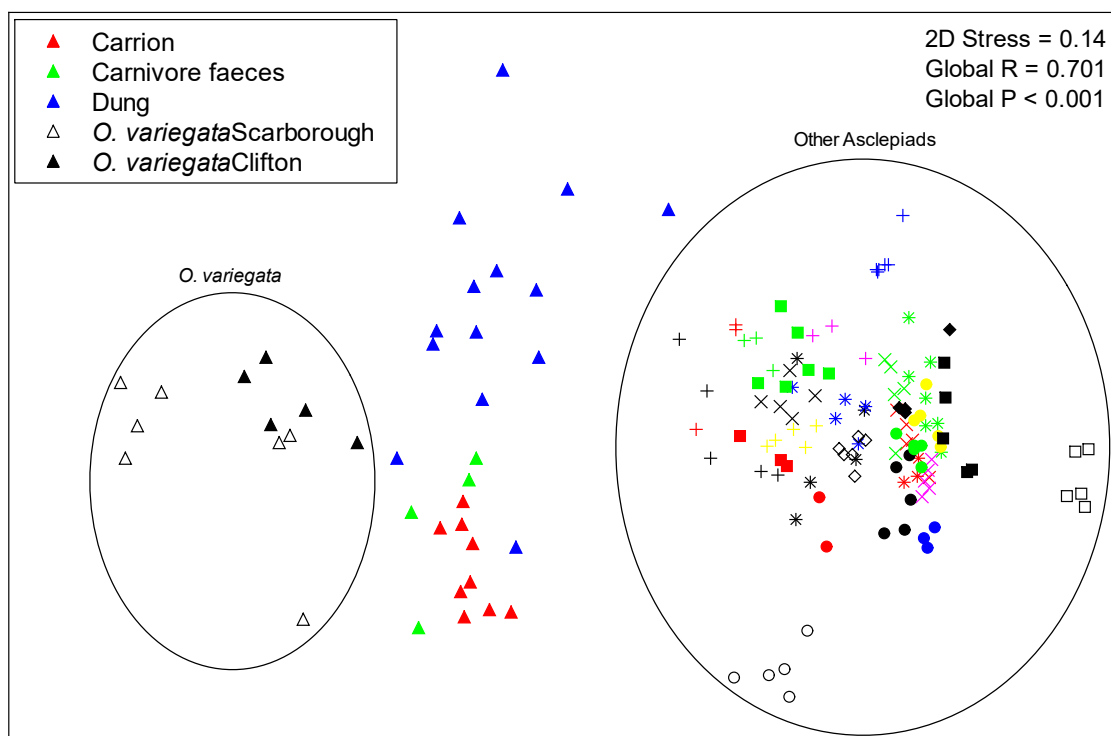

Figure 3. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis similarities of the odour composition of *O. variegata* flowers, carrion, carnivore faeces, dung and the floral scents of 26 species of asclepiads (each symbol represents samples from one species) (see Table 2) (asclepiad data from Shuttleworth & Johnson 2012; carrion and faeces data from Kheswa, N., Shuttleworth, A.

& Jürgens, A. unpublished data).

Table 8. Test statistics (R) resulting from pairwise ANOSIM contrasts comparing scent profiles of *O. variegata* flowers at both field sites to the scents of 26 species of asclepiads (see Table 2), carrion, carnivore faeces and herbivore dung. [a] = range of values due to pairwise comparison being done with each individual asclepiad species.

Sample	R value for *O. variegata* flowers from Clifton (p value)	R value for *O. variegata* flowers from Scarborough (p value)
Other asclepiads	All 1 (< 0.001 - 0.048)[a]	0.973 - 0.988 (< 0.001 - 0.028)[a]
Carrion	0.998 (< 0.001)	0.889 (< 0.001)
Carnivore faeces	0.919 (0.008)	0.696 (0.003)
Dung	0.451 (< 0.001)	0.694 (< 0.001)

NECTAR OF ORBEA VARIEGATA

No visible nectar was detected on *O. variegata* flowers from both field sites.

ANALYSIS OF FLORAL COLOURS AND THE COLOURS OF POTENTIAL MODELS

Two distinct colours can be perceived by the human eye on *O. variegata* flowers: the yellow on the corolla lobes and the outer corona and the dark maroon/black spots scattered over the corolla lobes and annulus. The yellowish background of the corona exhibited a dull spectrum (never exceeding 25%) with reflectance peaking in the UV and then increasing again from c. 500 nm. The yellow of the outer corona exhibited a similar spectral curve, but with slightly higher total reflectance. The dark red markings and the inner corona exhibit very low overall reflectance, but with reflectance increasing above 600 nm (Figure 4).
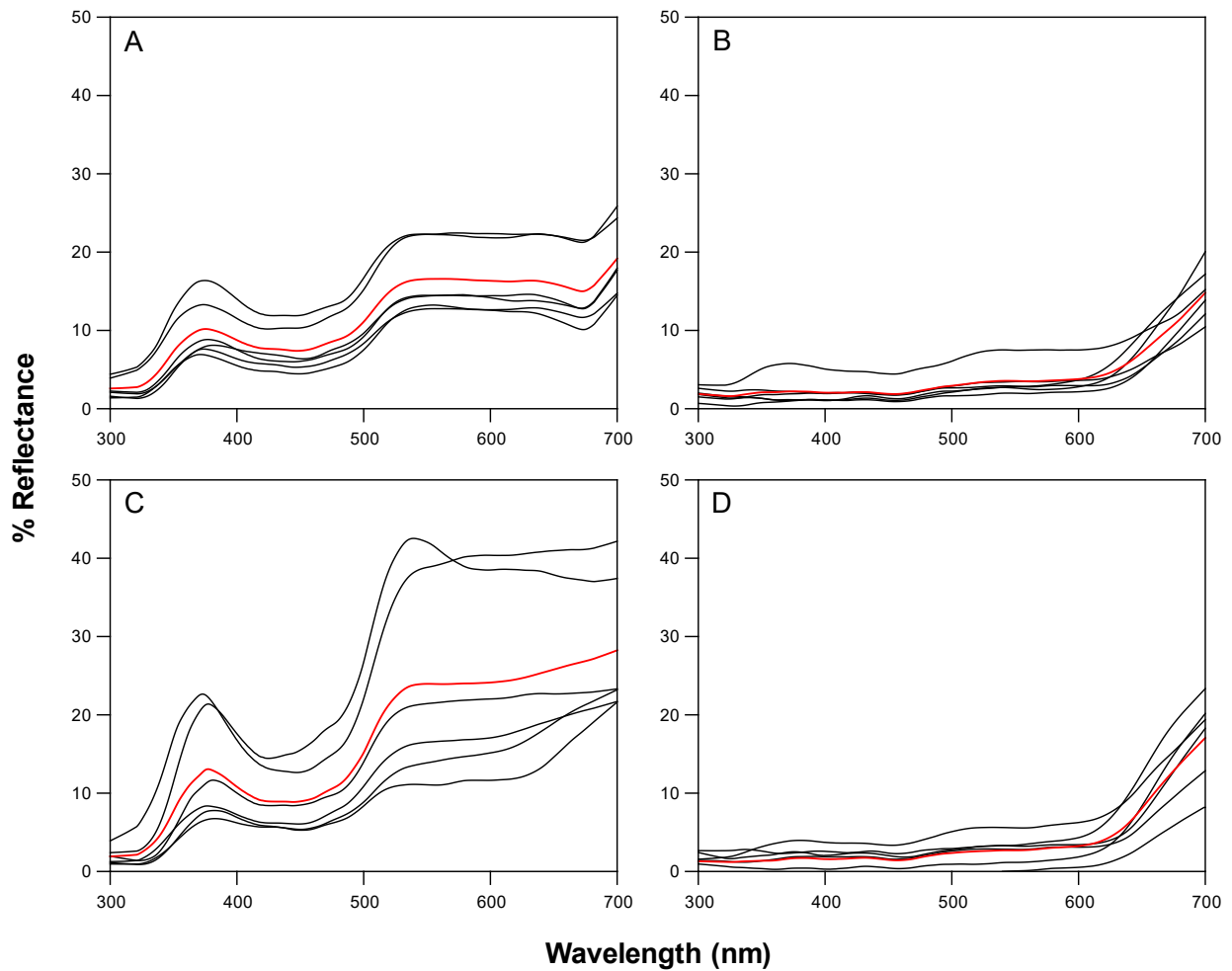
Figure 4. Reflectance spectra of different parts of six *O. variegata* flowers where the red line represents the mean spectrum of the six replicates. A: Yellow of corolla lobe, B: Black of corolla lobe, C: Yellow of outer corona, D: Black of outer corona.

The colours of the potential models also exhibited a dull spectrum which never exceed 10% reflectance. Both types of dung exhibited similar curves, with peaks in the blue, and then increasing again from 500nm. Rotting liver also exhibited a peak in the blue. The background rocks from the area exhibited relatively high spectral reflectance while increasing to approximately 600 nm with a slight plateau thereafter (Figure 5).
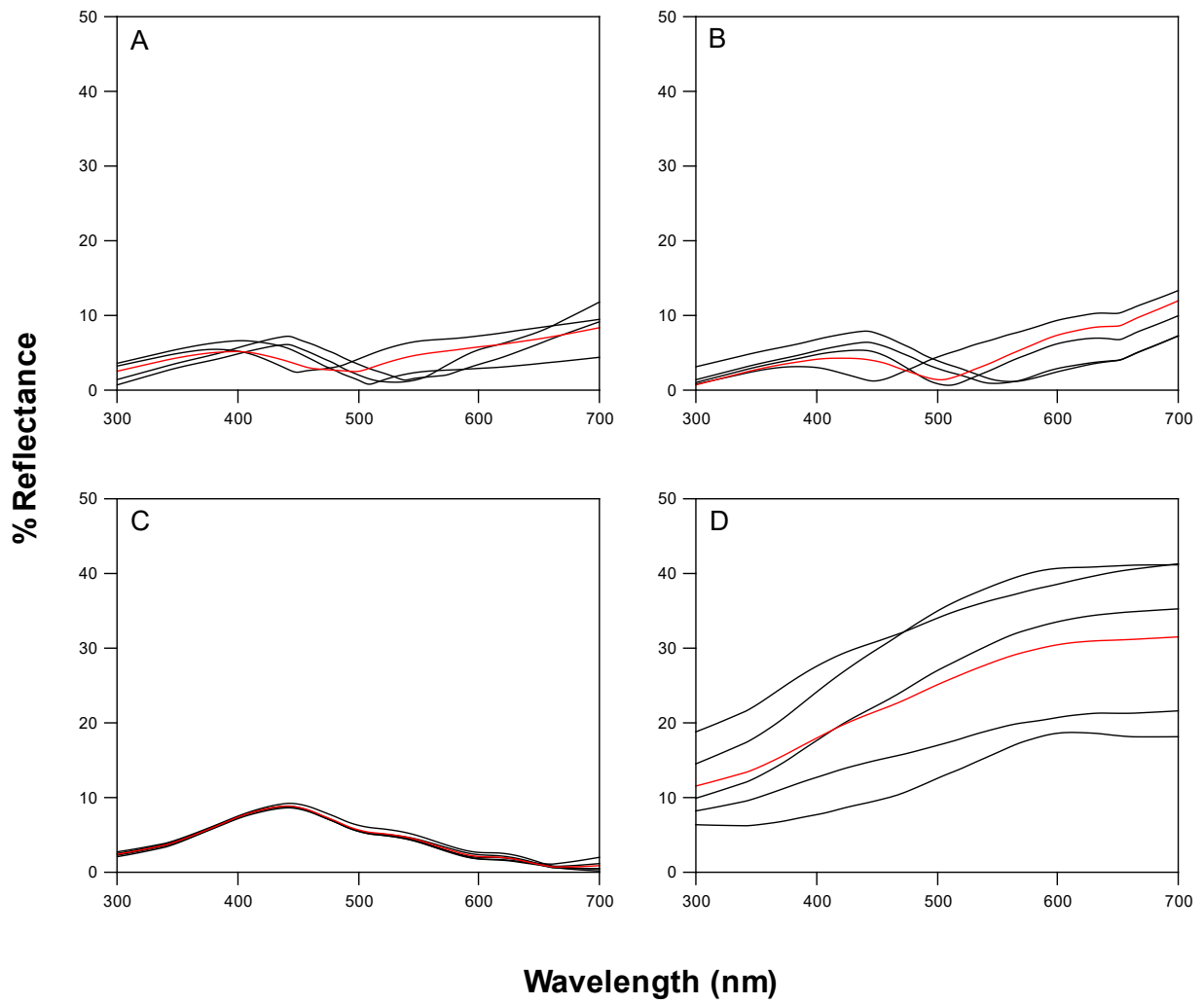
Figure 5. Reflectance spectra of different potential models as well as background rocks where the red line represents the mean spectrum of the six spectra. A: Horse dung, B: Sheep dung, C: Rotting liver, D: Background rocks.
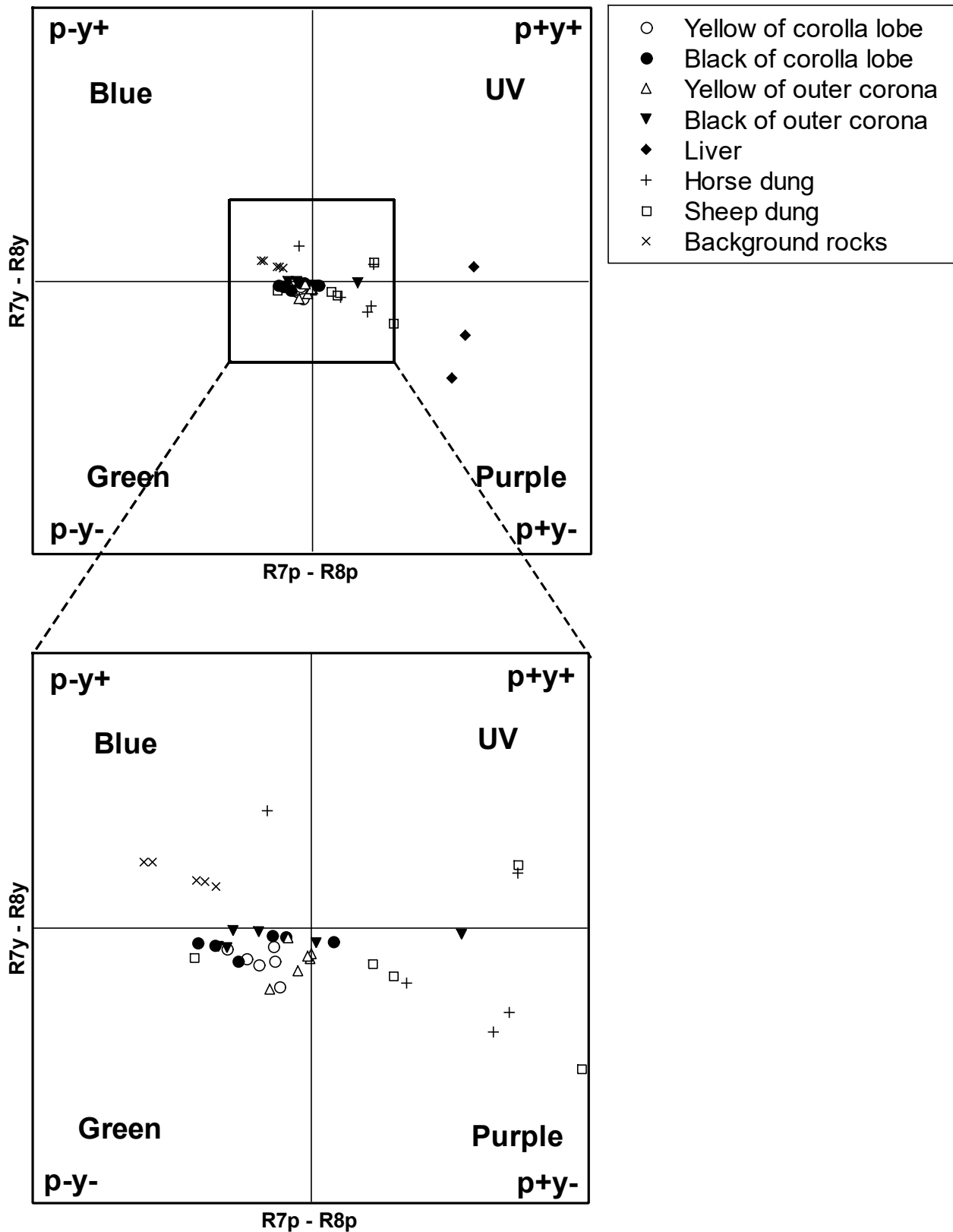
Figure 6. Colours of floral parts of six *O. variegata* flowers along with liver, dung and background rocks plotted as loci in the Troje (1993) fly colour vision model.

After implementing the fly vision model on the spectra of these different parts of the flower, most of these flower parts were perceived as fly-green, with some inconsistency in the black parts of the flower where they are sometimes perceived as fly-purple (Figure 6). The liver, horse dung as well as sheep dung was perceived either as fly-UV or fly-purple, and background rocks collected from the field site are all perceived as fly-blue (Figure 6).

EXPERIMENTS TESTING THE FUNCTIONAL SIGNIFICANCE OF VISUAL AND OLFACTORY SIGNALS

Flies were attracted to an *O. variegata* flower that was concealed from view (Figure 7). However, the exposed flower received significantly more visits than the concealed flower (Figure 7).
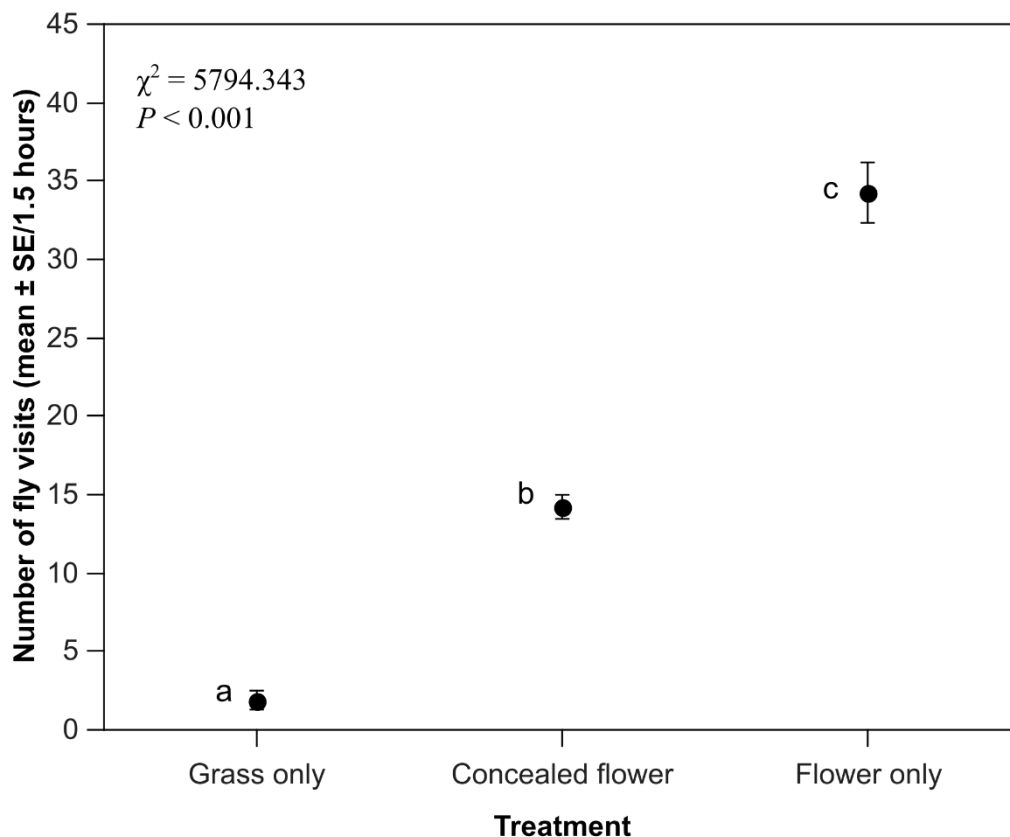

Figure 7. Mean number of fly visits to an exposed *O. variegata* flower, an *O. variegata* flower concealed with dried grass and a pile of dried grass without a flower (n = 5).
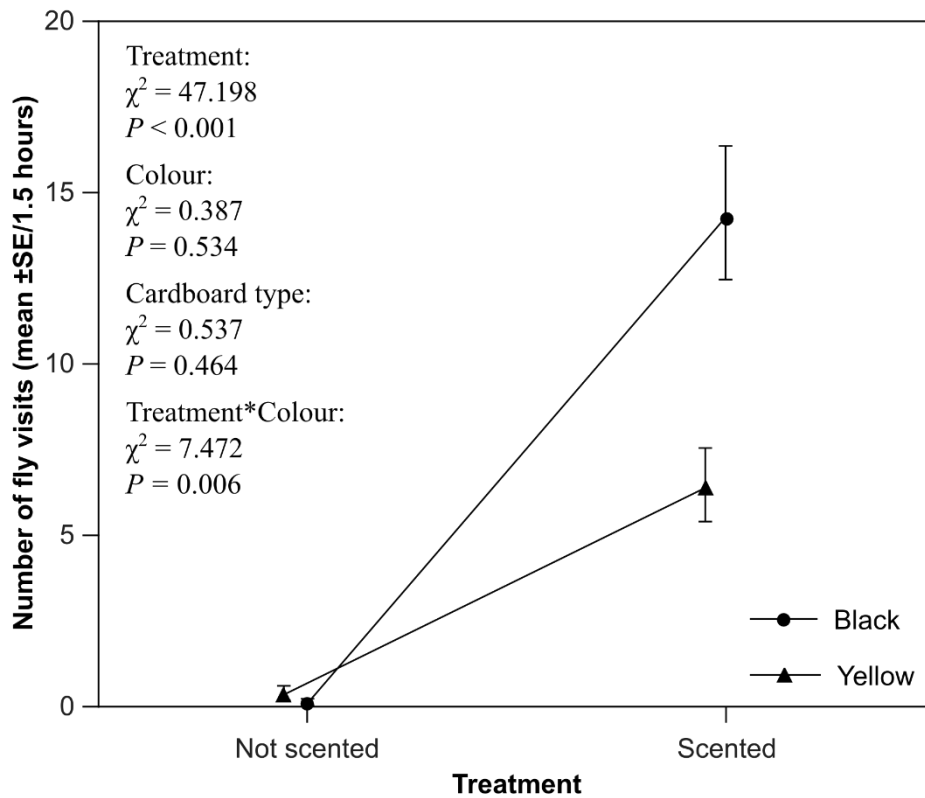
Figure 8. Number of fly visits to scented (with natural *O. variegata* odour) and unscented black and yellow cardboard model flowers (n = 10).
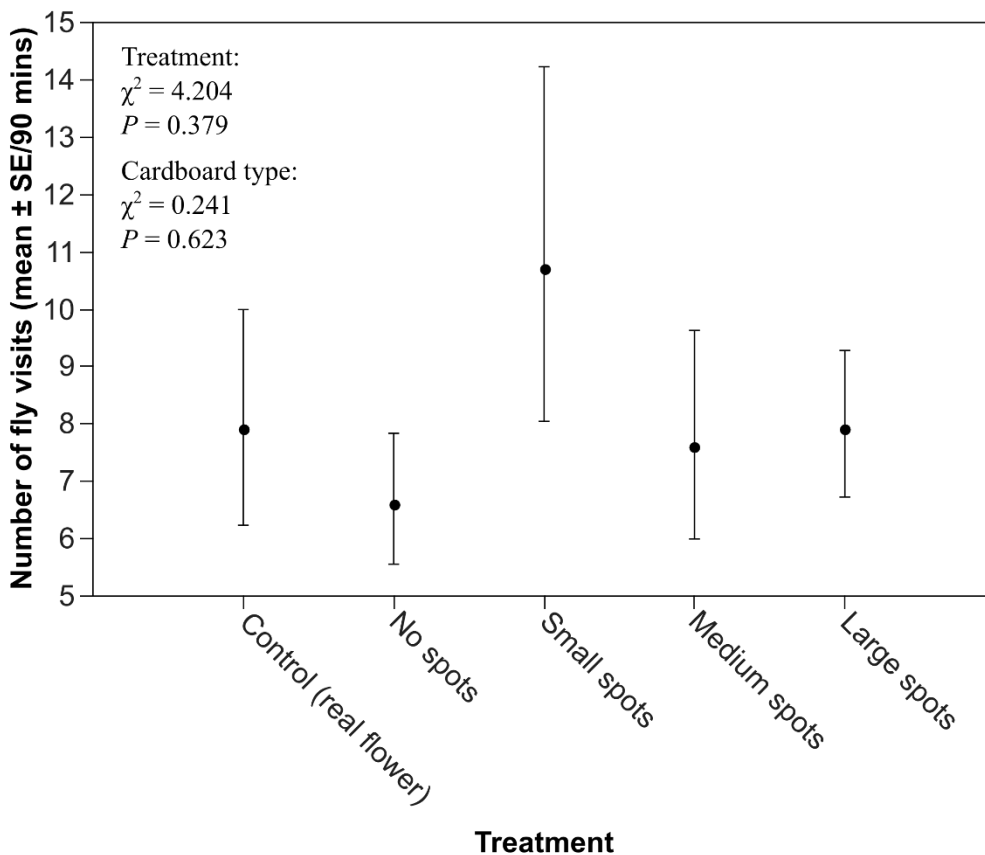

Figure 9. Mean number of fly visits to yellow cardboard model flowers with different sized blotching (scent provided by real *O. variegata* flowers) (n = 10).

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Black cardboard flowers in the presence of *O. variegata* odour received significantly more fly visits than a yellow cardboard flower in the presence of *O. variegata* odour (Figure 8). The size of the blotching on cardboard flowers had no significant effect on fly visits (Figure 9).
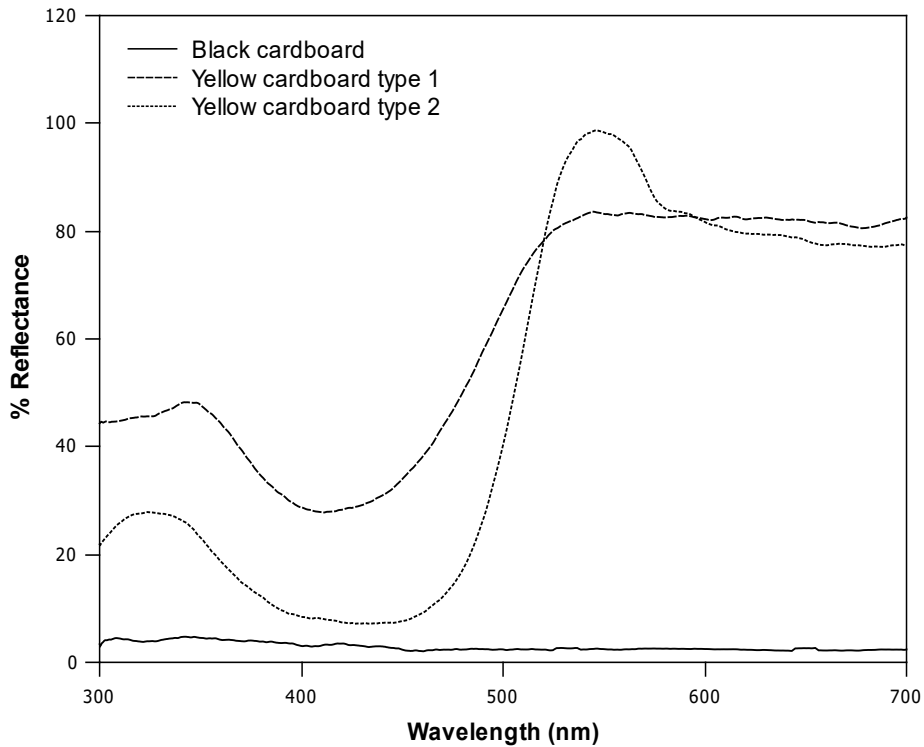

Figure 10. Reflectance spectra of cardboard used for fly choice experiments.

The black cardboard shows a typical black spectral reflectance (Figure 10). Two types of yellow cardboard were used, yellow cardboard type 2 used had a peak between 500 nm and 600 nm, whereas yellow cardboard type 1 did not exhibit as high a peak and is more similar in shape to the spectra of yellow parts of flowers (Figures 7). In terms of fly perception of these different types of cardboard, the black and yellow cardboard type 1 were perceived as fly-green, whereas the yellow cardboard type 2 was perceived as fly-purple (Figure 11).
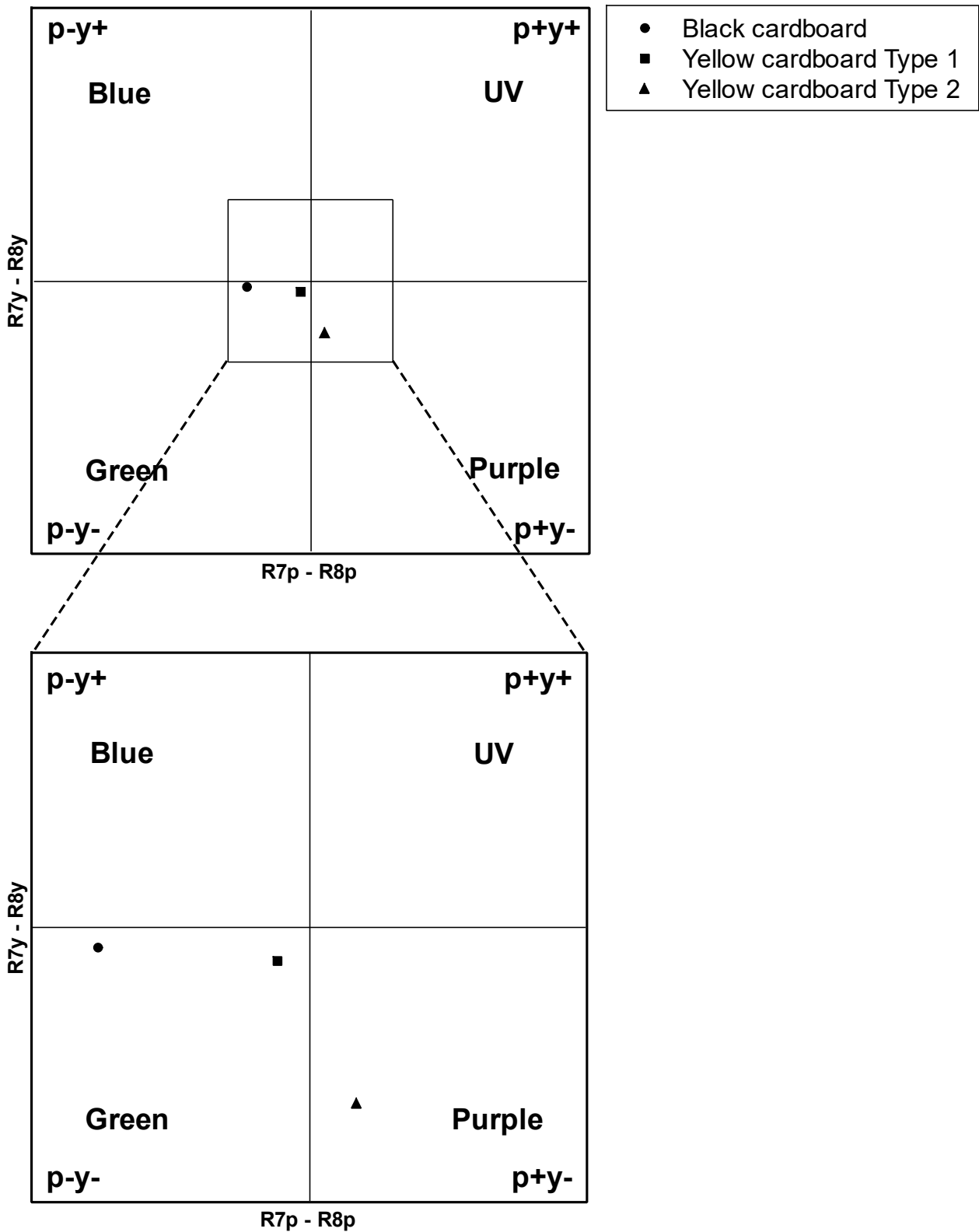
Figure 11. A: Colours of cardboard used as flowers during choice tests plotted as loci in the Troje (1993) fly colour vision model.

DISCUSSION

Orbea variegata is pollinated by flies from the families Calliphoridae (*Lucilia cuprina* and *L. sericata*), Muscidae (*Musca domesetica*) and Sarcophagidae. Pollinia were always attached to the mouthparts of flies (Table 3). Flies from the Muscidae family were the most abundant flies in terms of visitation rates across field sites (Table 4). The large majority of flies do in fact explore the centre of the flower, as well as most flies preferring the flower as a landing platform, as opposed to walking onto a flower (Figures 3 and 4). Muscidae were found to spend more time on flowers than do Calliphoridae (Figure 6). In this study, the addition of *O. variegata* scent to black and yellow coloured models supports previous findings that dark models are preferred by flies in the presence of putrid scents (Brodie et al. 2014; Kugler 1957; Wall & Fischer 2001). In addition, the dark patterning on the corolla lobes of *O. variegata* falls in the same quadrant as the background colour of the corolla lobes (Figure 6), and the size of the blotching pattern has no significant effect on fly visits (Figures 13 and 14). Visible flowers receive significantly higher fly visits than concealed flowers, reinforcing the importance of a visual component in the attraction of flies (Figure 7). Volatile emissions of *O. variegata* were dominated by the presence of phenol, dimethyl disulphide and dimethyl trisulphide (Tables 3 and 4). The NMDS analysis carried out as well as the ANOSIM analysis suggests that the scent of *O. variegata* flowers is more similar to dung than to carrion odour, although dimethyl trisulphide was also a dominant and characteristic compound which is normally associated with carrion; this implies that mimicry of both carrion and dung may be at play. These results supported my first two hypotheses, that flies would prefer black in the presence of carrion or dung, and that a visual cue needs to be present for odour source location.

Evidence from this study suggests that Sarcophagidae are the most effective pollinators (5 out of 6 collected Sarcophagidae flies were carrying pollinia), even though the number of Sarcophagidae collected was low (Table 3). It should be noted that Sarcophagidae were uncommon in the field which may explain the lack of observed visits by these flies to flowers. Although Calliphoridae and Muscidae were also found to carry pollinia, the numbers (carrying pollinia) were notably smaller when comparing the number of flies collected (1 out of 18 for Calliphoridae in 2015 and 4 out of 24 for Muscidae in 2015, Table 7). The morphology of Calliphoridae and Muscidae seem to not be perfectly suited to the size of the *O. variegata* guide rails, as a few individuals were observed escaping from the mechanism of attachment of a pollinium (pers. obs). This could be due to the morphology of the fly proboscis and the size of the *O. variegata* guide rails being better suited to each other in terms of size (Meve & Liede 1994) for the Sarcophagidae. However, some of the larger Muscidae

and Calliphoridae individuals were able to pick up pollinia. If one considers the abundance of Calliphoridae and Muscidae flies compared to the low numbers of Sarcophagidae, one could argue that Calliphoridae and Muscidae are equally effective to Sarcophagidae due to higher number of floral visitations (even if less efficient). It should be noted that 2016 was an extremely poor flowering season, with very few flowers developing, resulting in very few pollinators and none carrying pollinia. These results suggest that the pollination system of *O. variegata* is fairly generalist compared to that of other stapeliad members. Shuttleworth et al. (2017) showed that *Orbea lutea* subsp. *lutea* is highly specialised and pollinated by a single fly species from the genus *Atherigona* (Muscidae). Furthermore, *Stapelia gigantea* has been found to be pollinated exclusively by a few species of *Chrysomya* (Calliphoridae), even though it is visited by a range of carrion and dung associated flies from multiple families (Geers 2015). *Stapelia hirsuta* var. *baylissi* has been found to be pollinated mostly by a single species of *Anthomyia* (Anthomyiidae) and small contributions of Sarcophagidae, and is also visited by a range of different fly species that do not act as pollinators (Coombs 2010; Shuttleworth et al. 2017). The reason for the higher diversity of fly pollinators observed in *O. variegata* is not clear, although it likely relates to the dimensions of the guide rails and floral parts in relation to the sizes of locally available flies. Other stapeliads seem to achieve specialisation primarily by utilizing flies of particular sizes (Geers 2015). It is not always clear how different sized flies are filtered (Geers 2015), although *O. lutea* has been shown to rely on a morphological adaptation to prevent larger flies from accessing the nectar and making contact with guide rails (Shuttleworth et al. 2017).

The scent of *O. variegata* was dominated by the aromatic compound, phenol, as well as high relative amounts of sulphides (mostly dimethyl trisulphide) and aliphatics (albeit in varied relative amounts). The SIMPER analysis of the characteristic compounds of *O. variegata* scent composition, showed the three most characteristic compounds to be phenol, dimethyl disulphide and dimethyl trisulphide. Dimethyl disulphide and dimethyl trisulphide are usually emitted in similar amounts by carrion or dung mimicking flowers, such as the dead-horse arum (Stensmyr et al. 2002), two *Eucomis* species (Shuttleworth & Johnson 2010) and stapeliads (Jürgens et al 2006; Johnson & Jürgens 2010). Furthermore, Jürgens et al. (2013) found that both dimethyl disulphide and dimethyl trisulphide were present in similar relative amounts (means of 17.3% and 14.1% of the headspace samples respectively) in the scent profiles of 34 species of (what they suspected to be) oviposition site mimics. A mixture of dimethyl trisulphide and dimethyl disulphide has been shown to increase attraction of flies by Stensmyr et al. (2002) and subsequently also by Shuttleworth & Johnson (2010). Further, Zito et al. (2014), showed that dimethyl trisulphide elicited a stronger response than dimethyl disulphide in electroantennographic detection (EAD) using species of Muscidae (*Musca domestica*)

and Calliphoridae (*L. sericata*, *L. caesar*). This might explain the lower concentrations of dimethyl disulphide in the scent profile of *O. variegata*, as it is seemingly not as essential to the attraction of flies (of families Calliphoridae and Muscidae) as dimethyl trisulphide.

Comparing the NMDS of *O. variegata* scent to that of carrion, carnivore faeces, dung and other asclepiads showed that *O. variegata* scent is more similar to the carrion, carnivore faeces and dung than to other closely related plants (Figure 10). The pairwise ANOSIM suggests that the odour of *O. variegata* flowers is more similar to that of dung than carrion (Table 8). This could be due to the presence of phenol, *p*-cresol, benzyl alcohol and acetophenone, in *O. variegata* samples as they are generally prevalent in dung samples but not carrion or carnivore faeces (Jürgens et al. 2013). In addition, Kite et al. (1998) argue that the presence of phenol and skatole in floral scents are characteristic of dung mimicry, and interestingly the scent profile of *O. variegata* is partly dominated by phenol (Tables 3 and 4). The high relative abundance of phenol is also rather unusual, as phenol has thus far only been found in much lower relative amounts (Jürgens et al. 2006; Johnson & Jürgens 2010). The presence of dimethyl trisulphide suggests that there is also carrion mimicry at play. Dimethyl trisulphide is associated with protein decomposition (such as decaying flesh or carnivore faeces) (Jürgens & Shuttleworth 2015). This suggests that *O. variegata* in fact mimics both carrion and dung with no clear trade-off present. This strategy of mimicking both carrion and faeces could be advantageous as many adult flies use both carrion and dung as a source of food (Johnson & Schiestl 2016). Another aspect to keep in mind is that insects that are attracted to carrion often share similar morphologies to those that feed/breed on faeces, and therefore would be expected to offer similar functionality as pollen vectors (Johnson & Schiestl 2016). Since there does not seem to be a trade-off for *O. variegata* in mimicking both carrion and faeces, there is the advantage of attracting a larger quantity of functionally equivalent pollinators, as opposed to a smaller quantity of more specialised pollinators (Johnson & Schiestl 2016).

The floral colours of *O. variegata* are perceived by the human eye as yellow with deep red blotching. Substantial variety is present in the shade of the floral colours of *O. variegata*, where the yellow can range from very pale yellow to dark mustard yellow, and the black blotching can range from deep black to a dark maroon shade (pers. obs.). The size of the blotching on the lobes varies greatly in size. However, comparisons of how these colours are perceived by flies using the Troje (1993) fly vision model suggests that they are not different to a fly (Figure 6). This would mean that the flies perceive the yellow and the black patterning on the flowers as the same colour or that there is some achromatic contrast. Choice tests examining the blotching on lobes did not result in any significant changes to the number fo flies attracted (in different sized blotches as well as no blotching), this could only mean

that either the blotching is perceived (achromatically) and it has no effect on fly attraction, or blotching is not perceived at all. In terms of evolution, this would make sense as there is major variation in blotching, and since it does not affect fly attraction, there would be no selective pressure from the pollinator on specific blotching. Although, it may be possible that this variation in blotching size could specifically be selected for by factors that are not related to pollination, further studies would need to address any alternate functional significance of the blotching that is not pollination related.

Interestingly, horse dung, sheep dung as well as liver would be perceived as either fly-purple or fly-UV by flies. Therefore, it can be concluded that there is not a strong link in terms of colour or patterning with dung or carrion (Figure 6). It should be noted that the floral colours as perceived by flies would be distinguishable from the colour of the rocks they were growing amongst (Figure 6). Flies were also found to prefer black flowers over yellow flowers in the presence of *O. variegata* scent, with no difference in colour preference without scent (Figures 13 and 14). This result opposes some studies where flies have been found to prefer yellow models (Burg & Axtell 1984; Woodcock et al. 2014), although it does confirm Kugler's (1957) work. Kugler (1957) found that in the presence of carrion or faeces flies were found to prefer brown, purple-brown or even black before yellow models. These findings could be explained by the bimodal cue complex suggested by Brodie et al. (2014) where they found a preference for dark colours in the presence of dimethyl trisulphide and concluded that dimethyl trisulphide in addition to dark coloured models would represent a bimodal cue complex that would signify suitable oviposition sites.

Whether a fly lands directly on the flower (the odour source) or not poses an interesting question about the interaction of olfactory and visual cues. Olfactory cues can be considered to be a long distance attractant, whereas visual cues (colours, shapes and patterns) might only be useful for short distance orientation by the pollinator (Jürgens et al. 2006). It has been suggested that an important role of a flower is to represent a landing platform for a potential pollinator (Stebbins 1970), therefore, having a fly land directly onto the flower would showcase the plant's efficiency at guiding the fly to the odour source and providing a practical landing platform. In this study, I was able to provide evidence that *O. variegata* is very successful at aiding the flies in finding the odour source through visual cues as well as providing the before mentioned practical landing platform, as flies were found to mostly land directly on to the flowers (Figure 5). In addition, I found that concealing a flower has a significant impact on whether the flies are successfully able to locate the odour source (hence the flower). This is supported by the findings of Wall & Fisher (2001) as they also found that *L. sericata* (Calliphoridae) flies are able to locate an odour source more effectively with the addition of a target.

This shows that the synergy of visual and olfactory cue are likely to improve the chances of pollination, and it is likely that neither would be as successful on its own (Roy & Raguso 1997; Jürgens et al. 2006).

The behaviour of the fly on the flower (when it has successfully reached the flower) can also affect pollination success and efficiency. For *O. variegata* it is of immense importance that the fly does probe the gynostegium of the flower in order to pick up a pollinium due to the precise mechanisms of pollination, as well as to explore the gynostegium of another flower in order to insert the pollinium. This study provides evidence that most flies do indeed explore the gynostegium of the flower, with 92.5% of flies making it to the centre of the flower (Table 5), one possible motivation for flies to move to the gynostegium could be odour gradients, however, further studies would need to examine this possibility. Another fly behaviour that can impact the success rate of pollination is the amount of time that the flower is able to keep the fly on the flower. It makes logical sense to assume that the longer the fly stays on the flower probing the gynostegium, the higher the probability of pollination (or pollinium removal or insertion).

The percentage of flowers that set fruit was rather high, and was found to occur in 37.95% of flowers (with a mean of 169.8 seeds per follicle), which is higher than that reported for some other milkweeds (Shuttleworth & Johnson 2006; Shuttleworth & Johnson 2008; Shuttleworth & Johnson 2009b; Shuttleworth & Johnson 2009a) as well as *O. lutea* (Shuttleworth et al. 2017). Coombs (2010) reported a natural fruitset for *S. hirsuta* var. *bayliss*i never exceeded 8% over three seasons and Shuttleworth et al. (2017) reported a natural fruitset of 13.7% for *O. lutea* subsp. *lutea*. In contrast Herrera & Nassar (2009) reported 35% fruitset for *Stapelia gigantea* in Venezuela where it is invasive. The pollen transfer efficiency of *O. variegata* was 4.5% at Clifton and 9.5% at Scarborough, which is slightly lower but generally comparable to members of the Asclepiadoideae family which have pollen transfer efficiencies of around 20-30% (Harder & Johnson 2008; Shuttleworth et al. 2017). The PTE was also higher than that reported for *S. hirsuta* var. *baylissi and O. lutea* subsp. *lutea* (Coombs 2010; Shuttleworth 2016). Visitation rates were moderate for *O. variegata*, being 1.54 and 2.27 visits/flower/hour at field sites Clifton and Scarborough respectively.

This study has shown that *O. variegata* is pollinated by several carrion and dung associated fly species. My results suggest that the patterning on the corolla lobes of *O. variegata* has no effect on fly visits. This is rather interesting as this sort of patterning is not uncommon in carrion mimicking flowers. The purpose of blotching on the corolla lobes should be explored in further studies, and possibilities unrelated to pollination should be considered. It is also evident that flies prefer the colour

black in the context of *O. variegata*, therefore the reason why many carrion or faecal mimics (especially in the stapeliads) contain yellow or are completely yellow remains uncertain. There could once again be non-pollination related reasons for this such as thermal management and loss of moisture (as many of the stapeliads experience direct sunlight, such as *O. variegata*, in hot climates; pers. obs.). Mckee & Richards (1998) showed in their study on *Crocus chrysanthus* and *C. tommasinianus* that yellow coloured flowers exhibit the least floral warming compared to white and purple. Further studies would need to address functions of the yellow colouring often found in carrion or faecal mimicking flowers. The scent composition of these flowers is characterised strongly by the presence of dimethyl trisulphide and phenol, which is evidently sufficient in the attraction of carrion associated flies. Comparisons to models suggest that *O. variegata* mimics both carrion and dung, which means that carrion and faecal mimicking plants are not necessarily mimicking discrete categories. Rather it is possible to mimic both without clear trade-offs, which supports the suggestion of Johnson & Schiestl (2016) that there should not be any direct trade-offs for carrion or faecal mimicking plants as flies that feed on faeces and dung have similar functional morphologies. The effects of the visual cues of the flower has also demonstrated to be of immense importance, and the interaction between olfactory and visual cues appears to be the key to the success of this extraordinary plant.

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CHAPTER 3

Hairy and smelly: Functional significance of unusual floral traits in the attraction of flies by *Stapelia hirsuta* var. *hirsuta*

ABSTRACT

Carrion or faecal mimicking flowers are deployed by a number of angiosperms in order to attract, for pollination, insects that use carrion or faeces for feeding or oviposition sites. The flowers involved in these mimicry systems are reminiscent of carrion or faeces due to the similarities in volatiles emitted, the visual resemblance to these substrates as well as the position of the substrates (at ground level). Visual, olfactory and tactile features of these flowers are believed to be of importance in the attraction and manipulation of saprophilous insects, although there is limited empirical evidence for the functional significance of these traits. To evaluate the importance of the visual, olfactory and tactile traits exhibited by carrion and faecal mimicking flowers, I conducted a field-based study of the succulent putatively carrion-mimicking South African stapeliad *Stapelia hirsuta* var. *hirsuta*. These flowers attract flies belonging to the Calliphoridae, Muscidae and Sarcophagidae families, however, only members from Calliphoridae and Sarcophagidae were found carrying pollinia. This species naturally occurs in two distinct colour morphs: dull yellow colouring across the entire flower or a deep maroon with yellow patterning in the form of thin lines across the corolla lobes. The corolla lobes are generally covered in trichomes. The colours of these morphs were found to be indistinguishable from each other in a fly vision model. Flowers of both morphs were, however, chromatically distinguishable from the abiotic background and herbivore dung (a possible model), but were indistinguishable from rotten liver. The scent of both colour morphs was found to be dominated by dimethyl disulphide and dimethyl trisulphide, but the scent of yellow flowers also contained relatively large amounts of limonene while the scent of maroon flowers contained relatively large amounts of *p*-cresol. The yellow flowers received more fly visits than the maroon coloured morph. Flies could locate flowers concealed by grass, but not as easily as they did visible flowers. Lifting flowers off the ground had no impact on fly visits up to a height of 0.5 m; flowers lifted higher than that received fewer fly visits. Removal of floral trichomes of *S. hirsuta* resulted in fewer fly visits, as well as shorer periods of flower foraging by flies. This suggests that floral trichomes play a functional role in the attraction of flies. I conclude that the flowers of *S. hirsuta* are pollinated by

carrion associated flies and that floral trichomes and flower positioning affect the attraction and behaviour of flies.

INTRODUCTION

Carrion and faecal mimicking flowers are dependent on carrion associated insects for pollination, and their weird and unusual floral traits have often been assumed to play an important role in the attraction and manipulation of these insects (Johnson & Schiestl 2016). However, empirical evidence for the role of many of these bizarre traits remains scarce and the functional significance of features such as colour, scent, trichomes, papillae, vibratile clavate cilia and textured corollas remains poorly explored. Floral colour polymorphism is a feature that is also often associated with deceptive plants (particularly with food-deceptive plants) (Gigord et al 2001; Jersáková et al. 2009). Gigord et al. (2001) suggest that rare coloured morphs in deceptive systems may result in a reproductive advantage, however, any possible advantage from exhibiting different coloured morphs in the context of carrion or faecal mimicking flowers is unclear. Furthermore, geoflory is often a prominent feature in carrion and faecal mimicking plants (Johnson & Schiestl 2016) and may play an important role in pollination as the substrates that these flowers are mimicking are often situated at ground level. However, the importance of the flower positioning in the attraction of flies in carrion and faecal mimicking flowers has not been tested, and it should also be noted that not all carrion and faecal mimics exhibit geoflory (Bruyns 2005; Burgess et al. 2004). This study will explore these unusual traits in a putative carrion mimic.

One of the most well-known, and perhaps the best studied, aspects of carrion and faecal mimicry in plants is their scent chemistry. This has often been suggested to be rather specific in terms of matching assumed models. For example, Stensmyr et al. (2002) found that the scent emitted by the dead-horse arum (*Helicodiceros muscivorus*, Araceae) is very similar to the volatiles produced by dead gulls on which the plants pollinators (various carrion flies) oviposit. However, the odour composition of many of these flowers is also remarkably complex, with some species producing over 100 compounds (Jürgens et al. 2013). In a survey of the odours of stapeliads (Apocynaceae: Asclepiadoideae), a well-known group of foetid-smelling flowers, Jürgens et al. (2006) suggested that the compounds emitted by putative carrion and faecal mimics can be linked with specific models, such as herbivore dung (characterised by large relative amounts of p-cresol), carnivore dung and carrion (characterised by oligosulphides, heptanal and octanal) and, lastly, urine (characterised by various acids). Although it

makes sense for carrion and faecal mimics to closely match assumed models, Johnson & Schiestl (2016) pointed out that the odours of these plants do not always fall in discrete categories (of mimicry) and that these plants sometimes appear to mimic more than one model. According to Aigner (2001; 2004; 2006), the specialisation of a plant to one type of pollinator may reduce the effectiveness of another type of pollinator and therefore would represent a fitness trade-off for the plant. However, Johnson & Schiestl (2016) pointed out that the morphologies of the flies that use these different models for oviposition and feeding sites are often functionally similar, therefore a trade-off for mimicking multiple models would be unexpected. Although odour is quite well-studied, there are other traits, such as trichomes, which are also reminiscent of carrion and may also contribute to the overall mimicry.

Floral trichomes, the dense hairs which are often present on putatively carrion mimicking flowers (Faegri & Pijl 1979; Kite & Hetterscheid 1998; Jürgens & Shuttleworth 2015; Stensmyr et al. 2002) are particularly unusual and difficult to understand. The functional significance of floral trichomes has often been speculated on, and it has been suggested that the trichomes enhance the resemblance of the flower to the carcass of a dead mammal (Jürgens et al. 2006; Moré et al. 2013; Raguso 2004; Urru et al. 2011), or that the hairs may confer subtle hints of movement reminiscent of a carcass covered in flies and representing a suitable brood site (Vogel 1954). Bänziger (2001) argued that the presence of hairs on *Rhizanthes infanticida* (Rafflesiaceae) acts as a tactile cue stimulating oviposition by blowflies, although it remains unclear how this benefits the plant. Although the resemblance to a carcass makes intuitive sense, we have limited empirical evidence that trichomes enhance attraction of flies or manipulate fly behaviour on the flowers. Floral trichomes also appear to have different patterns of reflectance, such as UV reflection in *S. grandiflora* (A. Shuttleworth pers. comm.). In addition to floral trichomes, geoflory is a strategy also often (albeit not always) deployed by carrion and faecal mimicking plants (Bruyns 2005; Johnson & Schiestl 2016).

Geoflory is a floral feature that is often quite prominent in carrion and faecal mimicking plants and is also present in many stapeliads (Johnson & Schiestl 2016; pers. obs.). It has been suggested that geoflory is important in this system as the substrates they are mimicking are typically situated at ground level (Johnson & Schiestl 2016). This suggestion is based on the observation that many New World bat-pollinated flowers are known to produce some of the same sulphides as many carrion flowers (Bestmann et al. 1997; Knudsen & Tollsten 1995; von Helversen et al. 2000), but are not visited by flies. Carrion flowers, however, are not exclusively geoflorous (the flowers of *Hoodia* and *Aristolochia* species, for example, can be raised meters above the ground; Burgess et al. 2004; Bruyns 2005) and the prevalence of geoflory in these systems may be unrelated to the attraction of carrion-

associated insects. Although simple to test, the functional significance of flowers situated at ground level has not been explored in carrion-mimicking flowers and it remains unclear whether geoflory is an adaptation for the exploitation of carrion-associated insects.

Another unusual characteristic of many putative carrion or faecal mimicking species is the presence of extreme floral variation between individuals (Jürgens et al. 2013). Floral polymorphism is often associated with food-deceptive plants (Gigord et al. 2001; Jersáková et al. 2009). Polymorphism in animals has been suggested to have evolved as a reflection of the perceptive and cognitive characteristics of predators, as it would be much harder to develop a search image for multiple different prey forms than for a single prey morph. Predators thus focus on the most abundant forms giving an advantage to the less abundant forms which tend to be overlooked (Bond 2007; Gigord et al. 2001). This idea of negative-frequency dependant selection has also been suggested to explain floral polymorphism in rewardless flowers (Gigord et al. 2001). In this case, rare morphs would have a reproductive advantage over abundant morphs due to pollinators learning to avoid the common morphs (Gigord et al 2001). A similar explanation may explain the floral variation in carrion flowers. Some carrion flowers are rewardless, although there may also be other costs (such as the time wasted investigating an oviposition site which turns out to be a flower) which may result in pollinators learning to avoid these flowers and resulting in patterns of negative frequency dependant selection maintaining polymorphism within populations.

Stapeliads are generally well known for their foul odours and strange morphology (Urru et al. 2011; Jürgens et al. 2013). These succulent species generally occur in arid environments (Bruyns 2005). The foul odours emitted by these plants are believed to aid in the attraction of flies as pollinators (Jürgens et al. 2013). Many stapeliads have trichomes and vibratile clavate cilia on the perianth and corolla lobes, and as previously mentioned, the functional significance of these traits remains speculative. Stapeliads are also well known for their interesting colours and patterning, which often includes deep red and yellow, black and rarely green (Bruyns 2005; pers. obs.).

This study examines some of the unusual traits commonly associated with carrion and faecal mimicry using *S. hirsuta* var. *hirsuta* as a model species. The scent of *S. hirsuta* was first described by Sprengel (1783), where he suggested that the foul odour emitted by these flowers act as an attractant for blowflies and carrion flies for pollination (Johnson & Schiestl 2016; Vogel 1996). Interestingly, these suggestions by Sprengel (1783) represent the first mention of floral mimicry in plants (Johnson & Schiestl 2016). In terms of appearance, *S. hirsuta* var. *hirsuta* flowers are generally dark red or maroon in colour, with some bright yellow pattering in the form of thin lines on the corolla lobes (Figures 1

and 2). The flowers also have dense trichomes around the central areas, getting more sporadic on the corolla lobes where patterning occurs although trichomes do generally follow the edge of the corolla lobes in a thin line all the way to the tip of the lobe (Figures 1 and 2). The density of trichomes can vary hugely between plants. Interestingly, *S. hirsuta* var. *hirsuta* flowers also occur in a complete dull yellow floral colour, with slight patterning of a different shade of yellow (see Figure 2) (pers. obs.). The functional significance of these colours have not been explore in detail, although some case studies have been conducted in laboratory settings to determine the colour preferences of flies (Brodie 2014; Kugler 1957). The findings of these studies have not been consistent, although most have shown that flies prefer dark models in the presence of putrid scents and yellow or light coloured models in the presence of sweet scents (Kugler 1957; Brodie et al. 2014; Woodcock et al. 2014). This has not been tested in the context of faecal or carrion mimicking flowers.

The broad aims of this chapter are to establish the pollinators of *S. hirsuta var. hirsuta* and to explore the roles of the unusual floral traits in the attraction and manipulation of flies. The specific aims were to (1) describe the pollinators of *S. hirsuta var. hirsuta*, (2) quantitatively asses floral scent and colour, and compare these to those of putative models, (3) assess whether flowers offer a nectar reward, (4) explore the functional significance of scent, colour, trichomes and geoflory for the attraction of pollinators, and, (5) compare the traits and attractiveness of the two most frequent colour morphs (yellow versus maroon). I hypothesised that (1) the presence of trichomes on these flowers serve a dual purpose as visual signalling as well as tactile cues, (2) the yellow flowers will occur in a lower frequency than maroon flowers and therefore receive more fly visits, (3) that geoflory is a significant floral feature for the attraction of flies, and lastly (4) that *S. hirsuta* var. *hirsuta* mimics carrion.

METHODS AND MATERIALS

MODEL SPECIES AND STUDY SITE

This study was conducted on the succulent species *Stapelia hirsuta* var. *hirsuta* (N.E.Br.) Bruyns (Figures 1 and 2). *Stapelia hirsuta sensu* Bruyns (2000) includes five varieties. *Stapelia hirsuta* var. *hirsuta* differs from the other *S. hirsuta* varieties as it is one of only two varieties that have stems that are finely pubescent (Bruyns 2005). The other *S. hirsuta* variety with finely pubescent stems is *S. hirsuta* var. *gariepensis* (Bruyns 2005). *Stapelia hirsuta* var. *gariepensis* differs from *S. hirsuta* var. *hirsuta* in that the trichomes inside the corolla do not form a central cushion as well as the inner

corolla lobes being mottled with purple on yellow (Bruyns 2005). *Stapelia hirsuta* var. *hirsuta* is well known for its floral trichomes (hence the specific epithet), and often experiences fly oviposition on the flowers (Bruyns 2005; pers obs.). *Stapelia hirsuta* var. *hirsuta* has a broad distribution extending from Alexander Bay near the border of Namibia in the north, through Namaqualand to Vanrhynsdorp in the south (Bruyns 2005). In this area, it is commonly distributed along the western parts of the gneiss hills of the Kamiesberg from Springbok to Garies, and then more infrequently south towards the Knersvlakte (Bruyns 2005). Populations also exist around Malmesbury and Paarl and all the way to the western boundaries of the Little Karoo as well as the edges of the mountains to Prince Albert (Bruyns 2005). *Stapelia hirsuta* var. *hirsuta* can also be found further west on dry areas of the southern coastal plain from west of Greyton to Swellendam (Bruyns 2005). Throughout its distribution, *S. hirsuta* var. *hirsuta* occurs on rocky outcrops on mountains or among bushes on gentle slopes or flats (Bruyns 2005). The plant population used in this study was located on a private farm approximately 15 km east of Swellendam in the Western Cape (GPS: 34° 4' 14.2" S, 20 ° 33' 22.8" E, altitude: 128 m). This study site contained a population of c. 50 plants. All research was conducted in this population during the flowering season of 2016 (April and May). At this site, plants occurred between rocky outcrops on a gentle slope with some individuals scattered in between grasses at the top of the hill. Distinct colour morphs were observed to occur at the study site, with some individuals producing completely pale yellow flowers and other individuals producing very variable, deep maroon flowers with yellow patterning in the form of transverse lines; these yellow were mostly discrete with little variation, however, the colouring of the maroon morphs varied largely. The plants form dense clusters of erect almost parallel-sided stems, ranging in colour from unvaried green to green mottled with purple (Bruyns 2005). The *Stapelia hirsuta* var. *hirsuta* flowering season generally starts around the beginning of April and gets very sporadic at the start of June. Flowering peaked between mid-April and mid-May.
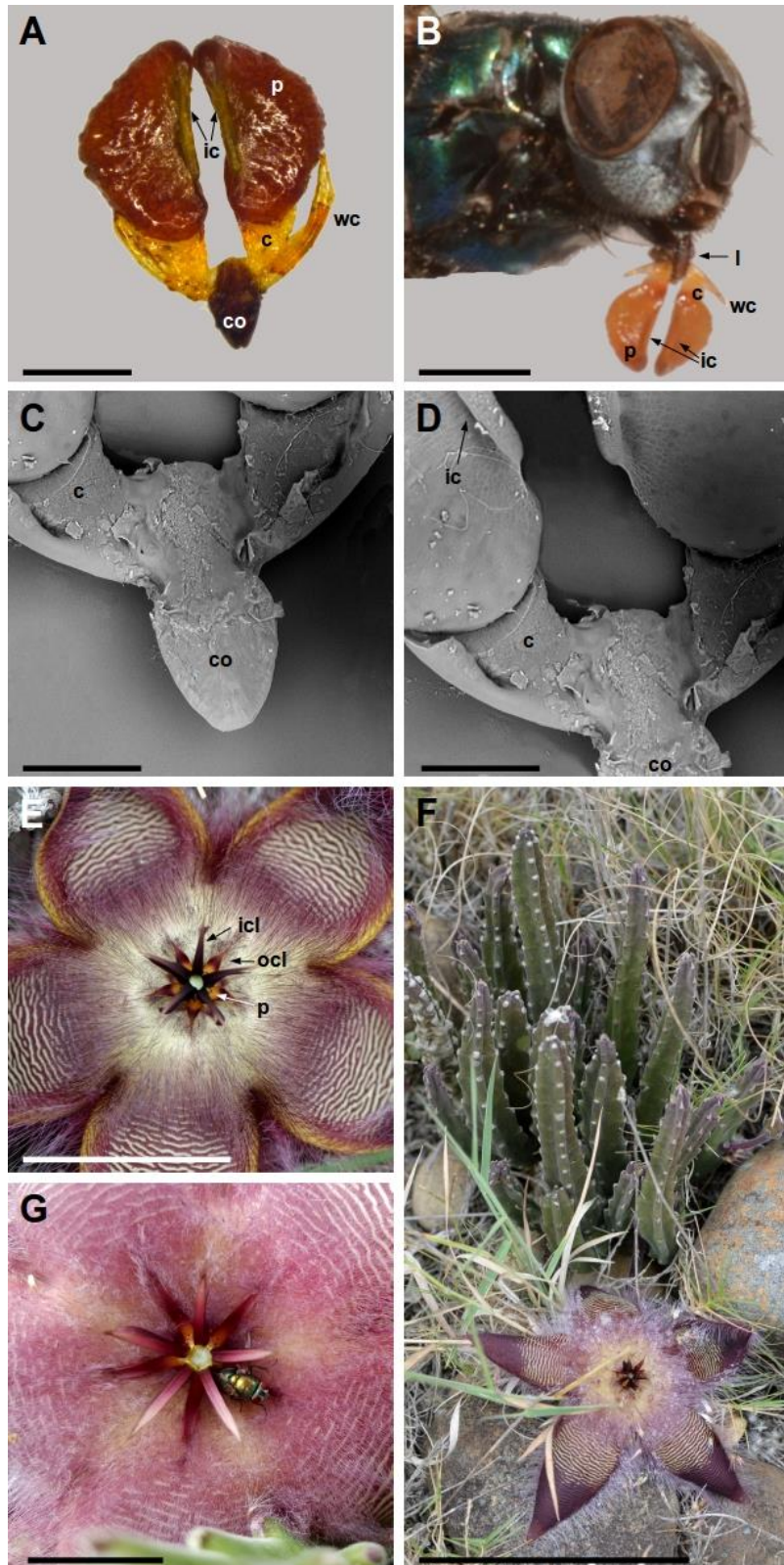
Figure 1. *Stapelia hirsuta* var. *hirsuta* and its pollinators **A.** Detail of pollinarium. Scale bar = 500 μm. **B.** Calliphorid fly with pollinarium attached to labium. Scale bar = 150 μm. **C.** Scanning electron microscope (SEM) image showing detail of corpuscle. Scale bar = 225 μm. **D.** SEM image showing detail of corpuscle and insertion crest. Scale bar = 225 μm. **E.** Close-up of a single flower. Scale bar = 4 cm. **F.** Whole plant with a single open flower. Scale bar = 7 cm. **G.** Calliphorid fly visiting a flower. Scale bar = 1 cm. (p) pollinium, (ic) insertion crest, (c) caudicle, (wc) wing of caudicle, (co) corpuscle, (l) labium, (icl) inner corolla lobe and (ocl) outer corolla lobe.
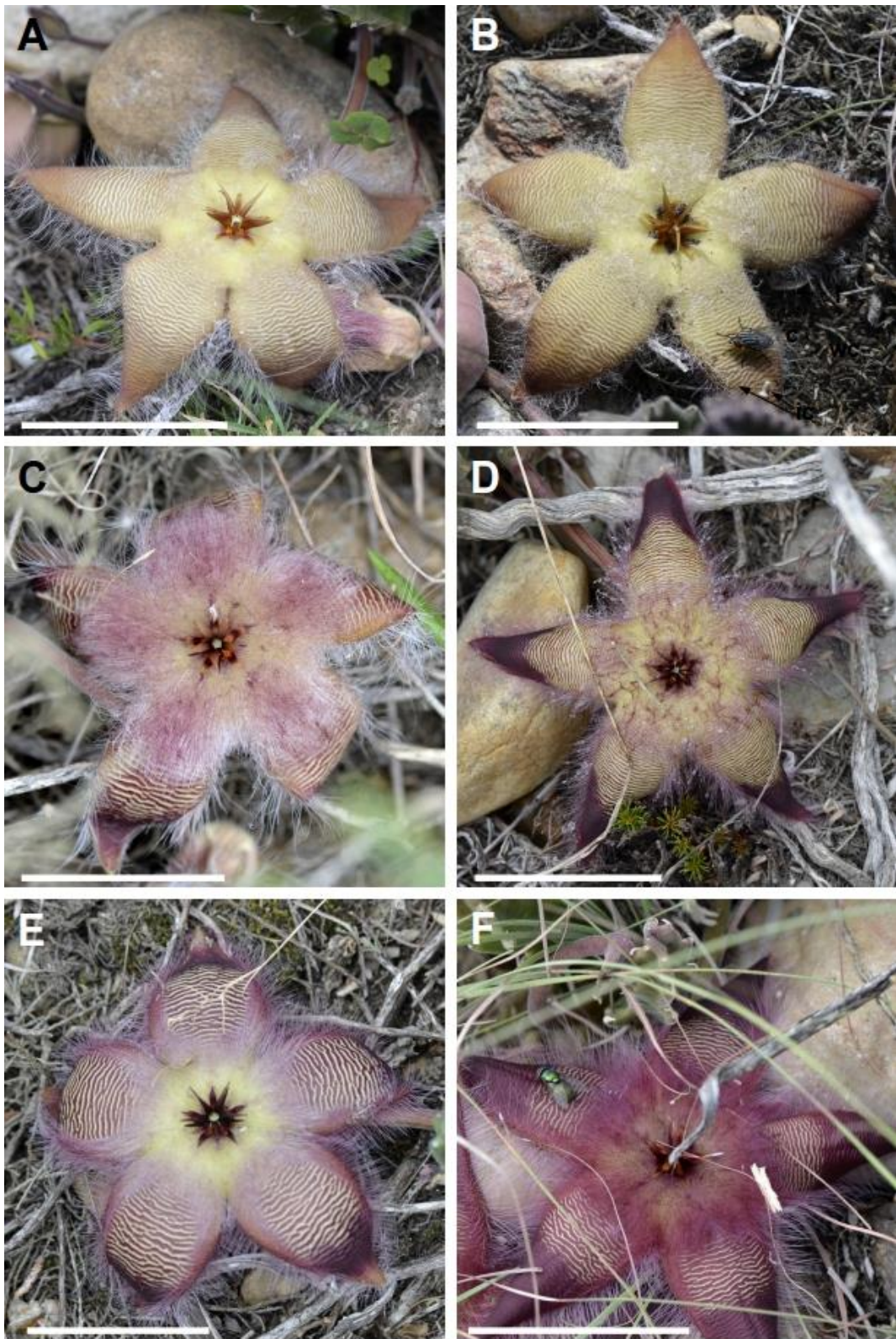
Figure 2. Floral variation of *S. hirsuta* var. *hirsuta* at Swellendam. All scale bars = 5cm.

FLORAL VISITORS AND THEIR BEHAVIOURS

Floral visitors were observed and representative specimens collected where possible. They were then pinned, identified (by N. Parry, University of Pretoria) and inspected for pollinia attached to body parts. Pollinators were observed for approximately 60 min per day over 12 days during the flowering season (commencing from 09h00 to 10h00). Differences in visitation rates and time on flowers were explored between the two colour morphs. Yellow morphs were defined as flowers containing no deep maroon colouring, whereas flowers that were predominantly maroon in colour were defined as the maroon morph. Even though the maroon morph always contained yellow patterning, maroon was always the dominant colour (Figure 1 & 2).

Behavioural experiments were conducted to determine how the flies behave on the flowers as well as how they arrive at the flowers. These experiments followed the same methods as the behavioural experiments of Chapter 2 determining how flies arrive at flowers and whether they move to the gynostegium.

FREQUENCY AND MICROHABITAT OF FLORAL COLOUR MORPHS

The frequency and microhabitat of both *S. hirsuta* floral morphs were surveyed to determine whether the different morphs could have arisen due to environmental factors. The flowers of 36 plants were surveyed and the colour recorded as well as the microhabitat (sun or shade). Any difference in colour within a single clone was also recorded.

POLLINATION SUCCESS AND NATURAL FRUIT SET

The pollen removal and insertion rates were scored for 96 open flowers (of varying ages). The pollen transfer efficiency (PTE) was calculated as the proportion of removed pollinia that were inserted into the guide rails.

To determine reproductive success of *S. hirsuta*, buds and flowers of 21 clones of different sizes were counted early during the flowering season in order determine the number of flowers per clone. Natural fruit and seed set were calculated using the same methodology as in Chapter 2.

FLORAL SCENT ANALYSIS AND COMPARISON WITH THE VOLATILES PRODUCED BY POTENTIAL MODELS

Floral volatiles were collected using dynamic headspace sampling methods following the same protocol as described in Chapter 2. Three yellow flowers and eight maroon flowers were cut (petiole was excluded from bag to reduce contamination by damage volatiles) and individually sampled for 90 min. A Similarity-Percentages (SIMPER) analysis was used to determine which volatile compounds best characterise the scent bouquet of *Stapelia hirsuta* var. *hirsuta* for both colour morphs. This analysis calculates the percentage contributions of each compound to average overall Bray-Curtis similarity between samples within a group (Clarke & Gorely 2006). A two-tailed t-test was conducted to determine whether any difference in emission rates exists between yellow and maroon flowers.

To explore the similarity between the odour of *S. hirsuta* flowers (of both morphs) and assumed models, I compared the odour to that of carrion, carnivore faeces and herbivore dung using non-metric multidimensional scaling (NMDS) as per methods described in Chapter 2 (see Table 1 in Chapter 2 for details of the carrion and faecal odour samples used). I also included the scent profiles of 26 non-carrion mimicking asclepiad species (obtained from Shuttleworth & Johnson 2012) in the NMDS analysis (see Table 2 in Chapter 2 for plant species used). Lastly, a one-way Analysis of Similarity (ANOSIM) was conducted (see Clarke & Gorley 2006) in Primer 6 (as per methods described in Chapter 2) to test for differences between the scent profiles of *S. hirsuta* flowers and each of the 26 asclepiads (Chapter 2, Table 2), as well as the carrion, carnivore faeces and dung odours.

NECTAR

Stapelia hirsuta flowers were inspected for nectar in the early mornings. A total of 30 flowers were inspected over the season.

ANALYSIS OF FLORAL COLOURS AND THE COLOURS OF POTENTIAL MODELS

Analysis of floral colours and the colours of potential models were conducted following methods and

equipment described in Chapter 2. Reflectance spectra were taken of both floral morphs, focusing on the following floral parts: corolla lobes, inner- and outer corona. Two yellow morph flowers and three maroon morph flowers were used, and the average of five measurements was used for each flower part. The floral colours of the two morphs and the potential models were compared using the Troje (1993) fly vision model as described in Chapter 2.

EXPERIMENTS TESTING THE FUNCTIONAL SIGNIFICANCE OF FLORAL TRAITS

To explore the relative importance of olfactory versus visual signals, I conducted an experiment in which flies were offered a choice between concealed and exposed flowers. This experiment followed the same methods described in Chapter 2. This experiment was repeated 10 times, each on a separate day. This experiment was conducted between 11h00 and 14h00 on sunny days between late April and late May 2016.

To explore the role of trichomes on *S. hirsuta* lobes, trichomes were removed from a flower, and visitation rates as well as the amount of time spent foraging on the flowers by pollinators were compared to an unmanipulated control flower. Only probing activities were recorded (the timer was stopped during non-probing activities). Probing activities were described as visitors actively seeking rewards by licking the flowers. Hairs were removed by carefully pulling them out without damaging the flowers. For each replicate, two flowers were selected from a single clone. Equal visitation rates were first established for 15 min before commencing the experiment. Trichomes underneath corolla lobes were removed from the unmanipulated flower to control for any physiological reaction the flower might undergo and any possible effects of damage volatiles as a result of the trichome removal. The flowers were observed for 90 mins per replicate (all replicates were done on separate days) and visits by flies (scored by family) were timed and scored. This experiment was replicated 9 times at mid-day during early May.

Stapelia hirsuta flowers are well known for extensive oviposition on the corolla lobes by carrion associated flies (Bruyns 2005). To explore the effects of different degrees of hairiness on oviposition, small plastic containers were baited with fish and chicken livers (a suitable substrate for inducing oviposition by carrion flies; C. Weldon pers. comm.) and covered with different types of fabrics (secured to the container covering the opening) and levels of oviposition on each was scored. The different types of fabric used were artificial sheep wool, a fabric resembling short hairs (approximately 4 mm long), a fabric resembling longer hairs (approximately 1.2 cm long) as well as

fleece. As a control measure all fabrics were black and open baited containers were put out as controls. The baited containers were placed in the field for three days at a time and eggs were counted every 24 hours. These experiments were conducted at the University of Pretoria experimental farm (GPS: 25° 44' 32.7" S 28° 15' 26.9" E) and in the University of KwaZulu-Natal botanical garden (GPS: 29° 37' 28.0" S 30° 24' 16.3" E) in Pietermaritzburg. Each fabric covered three baited containers per replicate, and this was replicated five times during October and November 2016.

To test the functional significance of geoflory in *S. hirsuta*, the height of flowers was artificially manipulated and the effects on fly visitation recorded. This was done by securing a 1 m broomstick firmly into the ground, followed by attaching *S. hirsuta* flowers (with a small piece of stem included) from the same clone to the bottom (0 m), middle (0.5 m) and top (1 m) using cable ties. Equal visitation was established for 15 min before commencement of the experiment. Flowers were observed and fly (by family) visits counted for 90 min per replicate (replicates done on separate days). Flowers were rotated every 15 min during each replicate. The experiment was repeated nine times.

STATISTICAL ANALYSES

Data were analysed using generalized estimating equations implemented in SPSS 23 (IBM Corp). Models that analysed proportions incorporated a binomial error distribution and logit link function, while those that analysed counts incorporated a Poisson distribution and log link function or linear distribution with an identity link function. Significance was tested using chi-square statistics (Wald and Generalised score) and the Sequential Sidak procedure was used for post-hoc tests. Means and standard errors presented in graphs were obtained by back-transformation from the logit or log scales.

RESULTS

FLORAL VISITORS AND THEIR BEHAVIOURS

The assemblage of flies collected on *Stapelia hirsuta* var. *hirsuta* flowers consisted of flies belonging to the families Calliphoridae, Muscidae and Sarcophagidae, however, only Calliphoridae and Sarcophagidae were found to carry pollinia (Table 1). Muscidae were notably less common than

Calliphoridae and Sarcophagidae, and Calliphoridae were the most common visitors. Calliphoridae and Muscidae were observed visiting the flowers and often probing the corolla lobes are well as the gynostegium. Sarcophagidae visitors were less active than the Calliphoridae and Muscidae, although they were also observed probing the gynostegium. The flies were often seen arriving on the corolla lobes, followed by brushing through the trichomes and probing to the gynostegium. To remove pollinia, the flies had to face the guide rails while probing a small cavity below the guide rails at the base of the gynostegium. While doing so, the labium was sometimes trapped between the guide rails and picked up a pollinarium as the fly withdrew (Figure 1B). This process did not take long, and the attachment of pollinia often happened within seconds of the fly's arrival at the gynostegium (pers. obs). In all observed cases, the individual struggled to free itself before flying off. One small dead Sarcophagidae fly was also collected one morning with its mouthparts still trapped between the guide rails where it had apparently died due to being unable to free itself.

Table 1. Visitor families collected on *S. hirsuta* flowers and plants as well as pollen loads at Swellendam. All pollinaria were attached to fly mouthparts.

Species	No. collected (no. carrying pollinia)	No. observed (no. seen removing pollinia)
Calliphoridae		
Chrysomya marginalis Wiedemann.	4 (0)	
Lucilia cuprina Wiedemann.	7 (1)	
Lucilia sericata Meigen.	8 (2)	
Unidentified Calliphoridae		180 (3)
Muscidae		
Musca domestica Linnaeus.	3 (0)	
Unidentified Muscidae		23 (0)
Sarcophagidae		
Sarcophagidae sp. 1	3 (1)	
Sarcophagidae sp. 2	8 (2)	
Unidentified Sarcophagidae		34 (2)

The visitation rates to different coloured morphs did not differ significantly, with yellow flowers receiving more fly visits overall than maroon flowers (Table 2) ($\chi^2 = 0.002$, $P = 0.967$). Interestingly, no significant differences were found in visitation rates between floral morphs for Muscidae ($\chi^2 = 613.1$, $P = 0.990$) and Sarcophagidae ($\chi^2 = 0.136$, $P = 0.986$). However, a significant difference was found for Calliphoridae ($\chi^2 = 0.043$, $P = 0.001$). Visitation rates between fly families also differed significantly, with Calliphoridae being the most abundant visitors (Table 2) ($\chi^2 = 11.217$, $P = 0.004$). No significant effect of colour morph ($\chi^2 = 0.122$, $P = 0.727$) or fly family ($\chi^2 = 3.127$, $P < 0.209$) was found in the amount of time the visitors spend probing flowers (Table 2).

Table 2. Mean fly visits per hour and the mean time spent probing on flowers for both colour morphs.

Family	Yellow	Maroon
Visits per hour		
Muscidae	0.7 ± 0.0 (n = 10)	1.1 ± 0.1 (n = 13)
Sarcophagidae	1.3 ± 0.1 (n = 18)	1.3 ± 0.1 (n = 17)
Calliphoridae	3.5 ± 0.2 (n = 82)	2.9 ± 0.2 (n = 96)
Time per flower (mins)		
Muscidae	2.1 ± 0.3 (n = 10)	2.2 ± 0.3 (n = 13)
Sarcophagidae	2.8 ± 0.2 (n = 18)	2.4 ± 0.3 (n = 16)
Calliphoridae	2.6 ± 0.1 (n = 82)	2.4 ± 0.1 (n = 96)

No difference was found between fly families in terms of the percentage of flies exploring the gynostegium of *S. hirsuta* (Table 3; $\chi^2 = 0.708$; $P = 0.702$,). In terms of the percentages of flies landing directly on flowers, Sarcophagidae were found to land on flowers significantly less often than Muscidae and Calliphoridae, while the latter two were not significantly different (Table 3; $\chi^2 = 12.074$, $P = 0.002$).

Table 3. Percentage of fly visitors that explore the gynostegium and the percentage of flies that land on flowers. Letters represent significance, values with different letters differ significantly at a 95% confidence interval.

Family	Percentage of flies that explored the gynostegium	Percentage of flies that land directly on the flower
Muscidae	67.1 ± 9.4 [a] (n = 23)	82.8 ± 7.9 [b] (n = 23)
Sarcophagidae	72.0 ± 6.8[a] (n = 34)	66.2 ± 6.5 [c] (n = 34)
Calliphoridae	65.6 ± 2.4 [a] (n = 180)	87.6 ± 1.7 [b] (n = 179)

FEQUENCY AND MICROHABITAT OF FLORAL COLOUR MORPHS

Of the 36 plants surveyed, 30 (83%) produced maroon flowers and only 6 (17%) produced yellow flowers. Of the maroon coloured flowers, 26 (87%) were growing in the sun and four (13%) were growing in shade. Of the six yellow coloured flowered, four (66%) were growing in sun and two (33%) were growing in shade. All individual plants produced flowers of the same colour.

POLLINATION SUCCESS AND NATURAL FRUIT SET

The pollen transfer efficiency (PTE) for *S. hirsuta* was 8.7%. The mean removal and insertion rates

were 3.5 ± 0.3 and 0.3 ± 0.0 pollinia per flower per clone respectively. Of the flowers inspected, 30.2% were pollinated (having at least on pollinium successfully inserted). The individuals surveyed for natural fruit and seed set produced 105 flowers in total. These flowers produced 46 fruits (47.6 %), of which four fruits were single follicles. The mean number of seeds per follicle was 270.1 ± 26.4 (n = 96), and the mean number of seeds per fruit was 518.7 ± 49.5 (n = 50).

FLORAL SCENT ANALYSIS AND ASSESSMENT OF THE VOLATILES PRODUCED BY POTENTIAL MODELS

The volatile headspace of *S. hirsuta* var. *hirsuta* was dominated by dimethyl disulphide and dimethyl trisulphide, with dimethyl disulphide (with one exception) having the higher relative amount (Table 4). *Stapelia hirsuta* flowers produced few compounds, ranging from 5 compounds to 7. The scent of the yellow morphs also contained high relative amounts of limonene, which was almost absent from the maroon morph, whereas the maroon morph contained much higher relative amounts of *p*-cresol than the yellow morph (Table 4). There was also a broad range of total volatiles (ng) emitted per flower per hour, ranging from 16.94 ng/flower/hour to 116.2 ng/flower/hour. A two-tailed t-test revealed that there is no significant difference in emission rates between yellow and maroon flowers ($p = 0.906$, $t = 0.122$). The SIMPER analysis identified dimethyl disulphide, dimethyl trisulphide and limonene as characterising the scent of the yellow morph flowers (Table 5). In contrast, the SIMPER analysis identified dimethyl disulphide, dimethyl trisulphide and *p*-cresol as characterizing the scent of maroon flowers (Table 5).

Table 4. Relative amounts (%, based on peak area) of volatiles identified from headspace samples of *S. hirsuta* at Swellendam in the Western Cape. tr = compounds that represent < 0.005% of total odour blend. Identification criteria: A = library match only, B = library match and confirmation with published retention index and C= library match and confirmation with synthetic standard.

| Compound | Kovats | ID Criteria | Yellow | | | Maroon | | | | | | | |
			Yellow 1	Yellow 2	Yellow 3	Maroon 1	Maroon 2	Maroon 3	Maroon 4	Maroon 5	Maroon 6	Maroon 7	Maroon 8
Aliphatic alcohols													
6-Ethyl-2-methylhept-6-en-2-ol	1369	A	-	6.55	-	-	-	-	-	-	-	-	-
Aromatic compounds													
Phenylacetone	1747	B	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
p-Cresol	2137	C	3.42	-	tr	31.68	1.41	16.40	18.94	6.83	6.80	5.29	9.98
Monoterpenes													
β-Pinene	1178	C	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
Limonene	1193	C	10.84	36.13	42.75	6.58	-	-	-	-	-	-	-
Nitrogen-containing compounds													
Indole	2504	C	3.58	1.59	1.82	-	-	7.29	14.91	1.54	3.18	10.62	5.44
Sulphur-containing compounds													
Dimethyl disulfide	1172	C	53.09	29.80	35.97	-	72.63	65.83	55.99	72.04	78.20	70.42	68.50
Dimethyl trisulfide	1377	C	29.06	25.93	19.46	55.60	25.96	10.48	10.15	19.59	11.82	13.67	16.09
Unknown compounds													
m/z 45,57,55,83,43,56,71,41	1257		-	-	-	6.14	-	-	-	-	-	-	-
Aliphatic compounds			-	6.55	-	-	-	-	-	-	-	-	-
Aromatic compounds			3.42	tr	tr	31.68	1.41	16.4	18.94	6.83	6.8	5.29	9.98
Monoterpenoids			10.84	36.13	42.75	6.58	tr	tr	tr	tr	tr	tr	tr
Nitrogen-containing compounds			3.58	1.59	1.82	-	-	7.29	14.91	1.54	3.18	10.62	5.44
Sulphur-containing compounds			82.15	55.73	55.43	55.60	98.59	76.31	66.14	91.63	90.02	84.10	84.58
Unknown compounds			-	-	-	6.14	-	-	-	-	-	-	-
Total number of compounds			7	7	7	6	5	6	6	6	6	6	6
Total volatiles (ng) emitted per flower per hour			29.37	69.33	33.07	21.20	116.94	116.2	16.94	25.19	19.83	24.67	36.69

Table 5. Compounds characterizing the odour of the two floral morphs of *S. hirsuta* based on a SIMPER analysis. Contribution represents the contribution of individual compounds to the first 90% of overall Bray-Curtis similarity within colour morphs of *S. hirsuta*. Sim/SD = % contribution divided by standard deviation. High percentage contributions and high Sim/SD values indicate compounds that best characterize the scent bouquet of the two colour morphs.

Compound	Sim/SD	Contribution (%)
Yellow flowers		
Dimethyl disulphide	12.99	35.48
Dimethyl trisulphide	16.61	29.31
Limonene	2.64	26.43
Maroon flowers		
Dimethyl disulphide	1.68	46.33
Dimethyl trisulphide	6.10	27.15
p-Cresol	2.94	18.08

The scent of *S. hirsuta* was closest to dung in a two-dimensional scent space (Figure 3). This was confirmed by the results of the ANOSIM pairwise comparison which showed that the floral scent composition of *S. hirsuta* flowers was most similar to that of dung (Table 6).
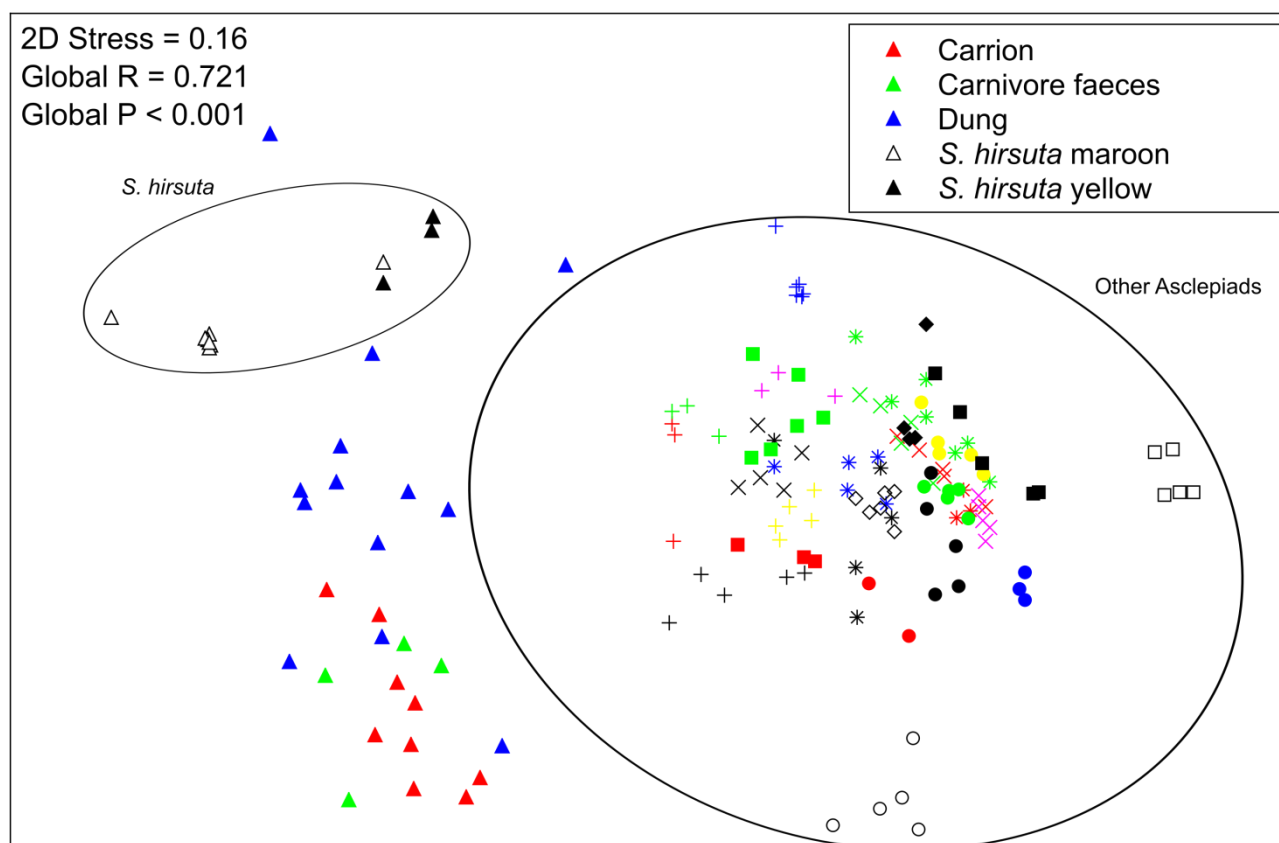

Figure 3. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarities of the odour composition of multiple samples of *S. hirsuta* var. *hirsuta*, carrion, carnivore faeces, dung and 26 other species of asclepiads (for the latter, each symbol represents one particular species) (see Table 1) (Shuttleworth & Johnson 2012, Kheswa, N., Shuttleworth, A. & Jürgens, A. unpublished data).

Table 6. Test statistics (R) resulting from pairwise ANOSIM contrasts comparing scent profiles of *S. hirsuta* var. *hirsuta* flowers to the scents of 26 species of asclepiads (see Table 1), carrion, carnivore faeces and herbivore dung. [a] = range of values obtained from pairwise contrasts between each individual asclepiad species.

Sample	Yellow *S. hirsuta* flowers (p value)	Maroon *S. hirsuta* flowers (p value)	Other asclepiads (p value)
Other asclepiads	0.988 – 1 (0.008 – 0.100)[a]	All 1 (< 0.001 – 0.022)[a]	-
Carrion	0.983 (0.005)	0.979 (0.003)	All 1 (< 0.001 – 0.18)[a]
Carnivore faeces	1 (0.029)	1 (0.002)	All 1 (0.003 – 0.029)[a]
Dung	0.690 (0.003)	0.552 (< 0.001)	0.087 – 0.972 (< 0.001 – 0.303)[a]

NECTAR

No visible nectar was found in *S. hirsuta* flowers at the field site in Swellendam.

ANALYSIS OF FLORAL COLOURS AND THE COLOURS OF POTENTIAL MODELS

The corolla and outer corona of yellow flowers exhibited similar spectral reflectance with dull (reflectance never exceeding 20%) and impure spectra (low chroma) peaking between 500 and 600nm (Figure 7). Inner corona lobes reflected more at the red end of the spectrum conferring the deep reddish colour to the human observer, but did not exceed 5%.

Maroon flowers also exhibited very low reflectance overall (never exceeding 20%), with the corolla and inner corona exhibiting a slight increase in reflectance at the red end of the spectrum (Figure 7). The flowers also exhibited low chroma for the inner (with one exception) and outer corona, while purer colours were present on the corolla lobes (Figure 7).
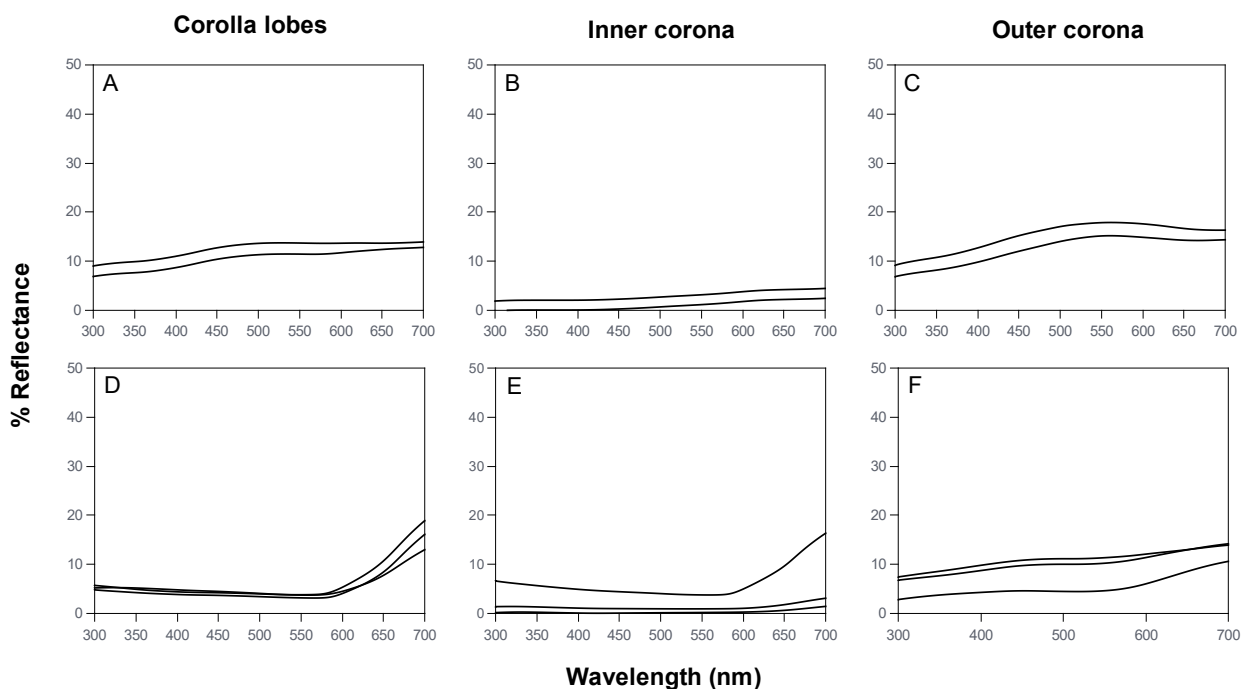
Figure 7. Reflectance spectra of different parts of two yellow (A – C) and three maroon (D – F) *S. hirsuta* flowers.

The colours of the two types of dung were very dull, with percentage reflectance lower than 10% for both (Figure 8). Both types of dung showed peaks in the blue and yellow regions of the spectrum resulting in a human-purple hue (Figure 8). Both types of dung exhibited impure spectra with low total reflectance. The spectral reflectance for rotting liver was very consistent with reflectance peaking between 400 and 500nm and limited reflectance at the red end of the spectrum (Figure 8). The brightness was once again very low, with the percentage reflectance staying below 10% (Figure 8). The colour spectra of the rocks were all very consistent in shape, but varied in brightness ranging from a percentage reflectance of 40% to lower than 10% (Figure 8). The rocks exhibited impure spectra increasing evenly from UV through to the red end of the spectrum (Figure 8).
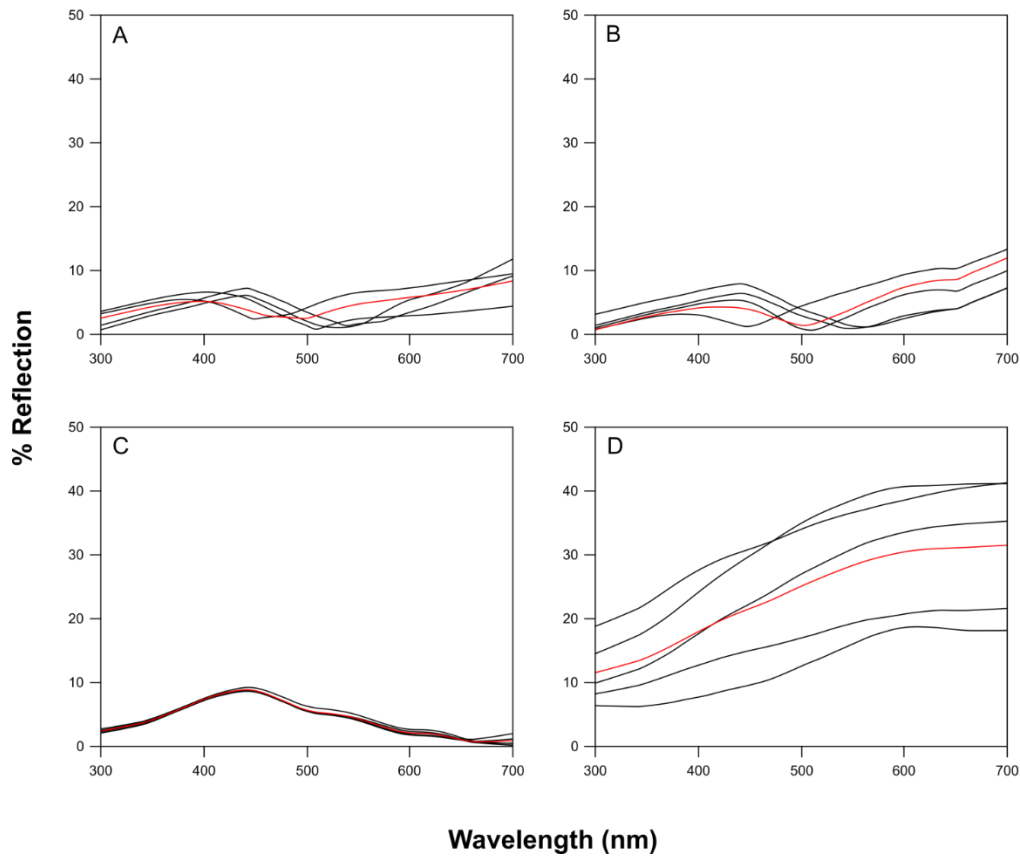
Figure 8. Reflectance spectra of different parts of potential models as well as background rocks where the red line represents the mean spectrum of the six replicates. A: Horse dung, B: Sheep dung, C: Rotting liver, D: Background rocks.

Loci for spectra for all floral parts of both morphs (yellow and maroon) fell in the blue quadrant of the Troje (1993) fly vision model (Figure 9). Liver and dung spectra were distributed in the blue and the purple quadrants, while background rocks were all in the green quadrant (Figure 9).
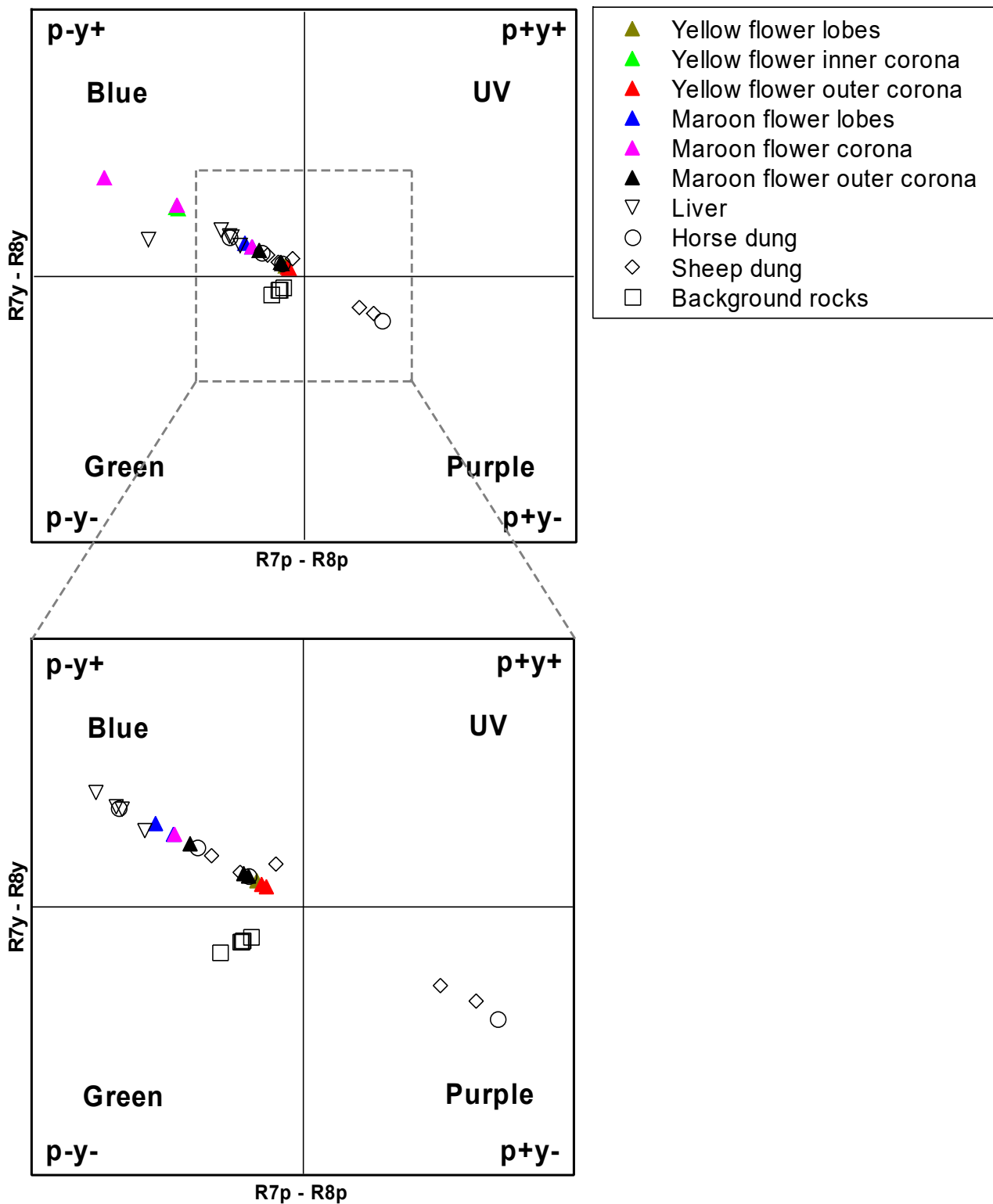
Figure 9. Colours of *S. hirsuta* flower parts along with liver, dung and background rocks plotted as loci in the Troje (1993) fly colour vision model. Bottom panel is a magnified section from the top panel.

The exposed *Stapelia hirsuta* flowers were visited significantly more by flies compared to the concealed flower and the grass, although, the concealed *S. hirsuta* flower did receive more visits than a pile of grass (Figure 10).
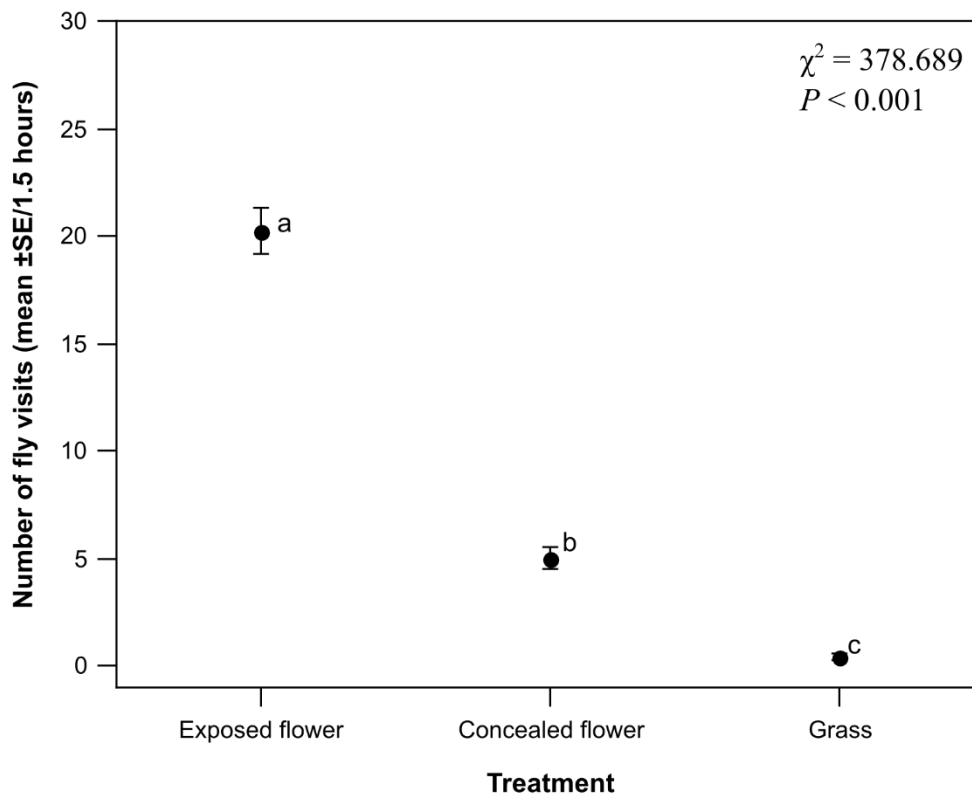

Figure 10. Mean number of fly visits to an exposed *S. hirsuta* var. *hirsuta* flower, an *S. hirsuta* var. *hirsuta* flower concealed with dried grass and a pile of dried grass without a flower. Data points with different letters differ significantly (n = 10).

The removal of the trichomes from the lobes of *S. hirsuta* flowers significantly decreased both the number of fly visits and the amount of time flies spent probing flowers (Figure 11).
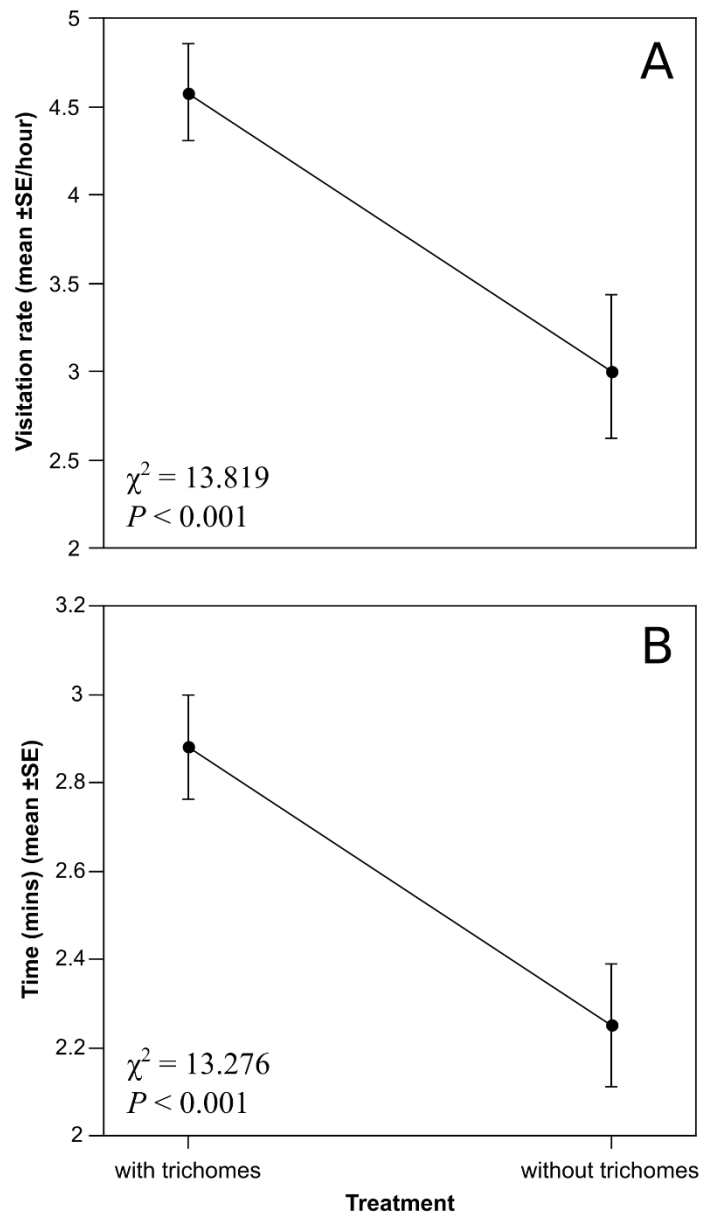
Figure 11. The effect of *S. hirsuta* trichome removal on (A) fly visitation rate and (B) the amount of time visitors spent probing flowers (n = 9).

No oviposition occurred on any of the fabric models or controls for all repetitions.

The height of the flower had a significant impact on the number of visits the flower receives. Although no difference was found in the number of fly visits at heights from the ground (0 m) up to a 0.5 m elevation, the number of fly visits decreased significantly for the highest (1 m) flower (Figure 12).
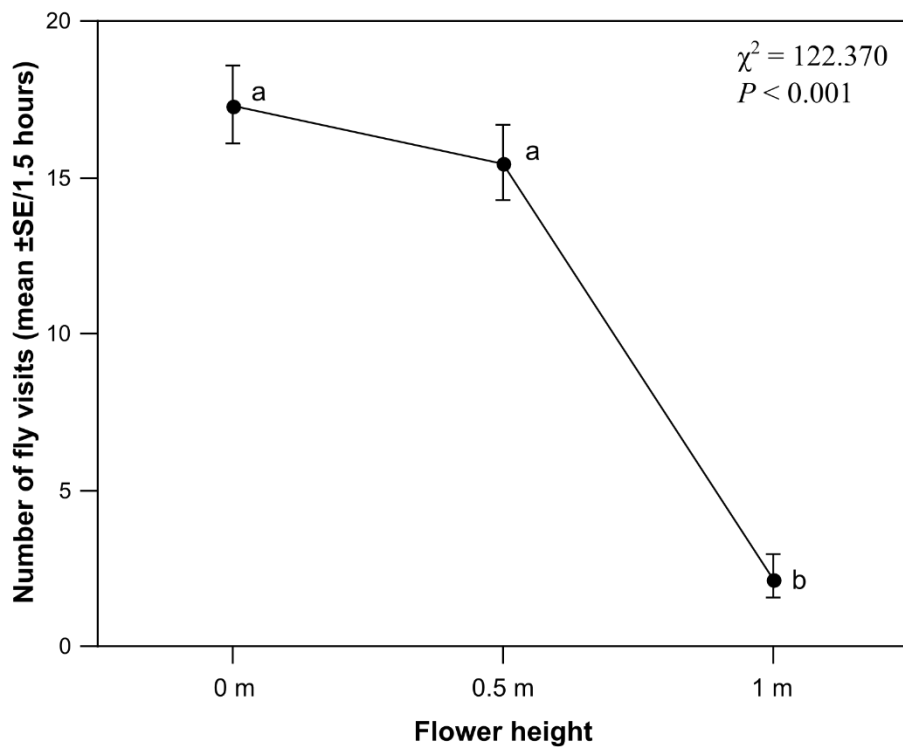
Figure 12. Fly visits to *S. hirsuta* flowers positioned at different heights. Data points with different letters differ significantly (n = 9).

DISCUSSION

This study found that *Stapelia hirsuta* var. *hirsuta* flowers are visited by various flies from the families Calliphoridae, Muscidae and Sarcophagidae at Swellendam. Of these, only Calliphoridae (two *Lucilia* species) and Sarcophagidae (two species) were found to carry pollinaria, which were always attached to the mouthparts (Table 2). Calliphoridae were the most abundant visitors to both morphs, with the yellow flowers generally receiving higher visitation rates than the maroon morph (Table 1). There is also evidence that the flowers are effective at guiding the flies to the gynostegium as the majority of flies exhibited this behaviour (Table 3). Both floral morphs' scent profiles were dominated by dimethyl disulphide and dimethyl trisulphide (Tables 3 and 4), however, the scent of yellow morph flowers was also characterised by limonene while the scent of maroon morph flowers was also characterised instead by *p*-cresol (Table 2). The NMDS suggests that *S. hirsuta* var. *hirsuta* is a dung mimic rather than a carrion mimic (Table 3), although the presence of both dimethyl disulphide and dimethyl trisulphide indicates that carrion mimicry is also present as these compounds are associated with decomposing carrion (Paczkowski & Schütz 2011). According to the Troje (1993) fly vision model, the maroon flowers and yellow flowers are chromatically indistinguishable by flies, but flowers were different from the abiotic substrates on which they grow (Figure 9). Flies did appear to

rely partly on visual cues when locating flowers as exposed flowers received significantly more visits than concealed flowers in an experiment comparing visitation rates to exposed versus concealed flowers (Figure 10). The removal of trichomes from the surface of the corolla was found to decrease fly visitation compared to control flowers on which trichomes were removed only from the underside of the corolla (Figure 11). Flies also spent less time probing flowers with hairs removed compared to control flowers (Figure 11). Fly visitation was also influenced by the situation of the flowers and flowers that were raised above 0.5 m received significantly fewer visits compared to control flowers situated at ground level and flowers placed 50cm above the ground (Figure 12). Therefore, my results support the hypotheses that trichomes serve a dual purpose as visual and tactile cues, that geoflory is an effective strategy for fly attraction and, and partlt that yellow morphs would attract more flies than the maroon morphs (as yellow flowers were more attractive to Calliphoridae, the most abundant visitors).

From the pollinator assemblages and pollen loads, there is no evidence that Muscidae are pollinators. Even though they do visit the flowers, they occur in much lower numbers than Sarcophagidae and Calliphoridae and were never observed to remove or carry pollinaria (Table 2, Table 2). This could be due to a mismatch between morphology of the *S. hirsuta* guide rails and the mouthparts of local Muscidae (Meve & Liede 1994). Shuttleworth et al. (2017) showed in their study on *Orbea lutea* subsp. *lutea* that even though the flowers attracted an array of flies, a morphological filter in the form of a narrow aperture prevented large flies getting to the pollinarium, resulting in only one fly species being able to pollinate the flowers. *Stapelia hirsuta* var. *hirsuta* does not have a physical mechanism like that of *O. lutea* to prevent a subset of potential pollinators from pollinating, although smaller flies may slip through the openings of guide rails without picking up pollinia, which could represent a less extreme filter allowing pollination only by larger flies. In further support, the visiting Muscidae flies were generally smaller than the Calliphoridae flies and Sarcophagidae flies (pers. obs.). A similar finding has been reported for *S. gigantea* which is pollinated primarily by a particular size-class of *Chrysomya* (Calliphoridae) flies (Geers 2015). Coombs (2010) found in a study on *S. hirsuta* var. *baylissi*, that this variety is pollinated by only a few species of Anthomyiidae flies. The findings of this study indicate that two species of Calliphoridae *(Lucilia sericata* and *L. cuprina*) and two species of Sarcophagidae are equally effective at pollinating the flowers, although it is apparent from the fly assemblage (Table 2) that Sarcophagidae are more efficient pollinators due to their higher pollen loads. These findings suggest that *S. hirsuta var. hirsuta* is relatively specialized and pollinated only by specific carrion-flies. This contradicts the popular notion that *S. hirsuta* flowers rely on any local carrion flies and supports a growing body of literature to suggest that stapeliads exhibit fine-scale patterns of specialisation to particular flies (Coombs 2010; Geers 2015; Shuttleworth et al. 2017).

The mechanisms of specialization are known to include both morphological adaptations (Shuttleworth et al. 2017) and selectively attractant odours (Geers 2015, Shuttleworth et al. 2017), but remain poorly understood for most species. The attraction of various saprophilous flies to these flowers was expected given the odour of these flowers.

The scent profiles of both *S. hirsuta* flower morphs were dominated by sulphur containing compounds, with dimethyl disulphide generally occurring in higher relative amounts (Table 2). These sulphides are well-established fly attractants and their functional significance in the attraction of flies to flowers has been established for several species (Shuttleworth and Johnson 2010; Stensmeyr et al. 2002; van der Niet et al. 2011). Interestingly, Zito et al. (2014) found that only dimethyl trisulphide (but not dimethyl disulphide) elicits an electroantennographic response from *Musca domestica* and certain species of Calliphoridae (notably *Lucilia sericata* and *L. caesar*). Subsequent behavioural experiments with these compounds suggested that DMTS was solely responsible for attracting these flies (Zito et al. 2014). However, Borg-Karlson et al. (1994) have shown in a field based study that *Calliphora*, *Lucilia* and *Sarcophaga* flies are attracted to dimethyl disulphide. Although these findings do contradict the findings of Zito et al. (2014), they suggest that the presence of dimethyl disulphide could allow for the attraction of a larger subset of flies. Interestingly the yellow morph was also characterised by limonene (which has a sweet citrus-like scent) and the maroon morph was characterised by *p*-cresol. Not many studies have been conducted to determine the functional significance of *p*-cresol in the attraction of flies. However, Jeanbourquin & Guerin (2007) have shown that *p*-cresol acts as an attractant for *Stomoxys calcitrans* (L) (Muscidae). Further, Birkett et al. (2004) have shown that *p*-cresol elicits an electroantennographic response by *S. calcitrans* and *Hydroteae irritans* (L.) (Muscidae). Notably, that the scent profile of the yellow morph exhibited greater similarity to other related asclepiads compared to the maroon morph when considering the ANOSIM analysis (Table 3) and the NMDS, and interestingly, one of the asclepiads, *Xysmalobium parviflorum*, has a scent that is dominated by *p*-cresol (Figure 7) (Shuttleworth & Johnson 2012). In contrast, limonene is a compound more commonly associated with flowers emitting fruity odours, as described for some stapeliads (Meve et al. 2004). However, Jürgens et al. (2013) found limonene to be present in the volatile emissions of horse, cattle and pig dung, as well as a dead vertebrate. Furthermore, Johnson & Jürgens (2010) found limonene to be present in a very high relative abundance (39 %) in horse dung. Johnson & Jürgens (2010) did not find limonene to be present in any of the stapeliads they sampled, although limonene was also present in *Ferraria crispa* Burm. (Iridaceae) which is also visited by members from Calliphoridae and Sarcophagidae (Johnson & Jürgens 2010). Limonene has not been reported to elicit an electroantennographic response in Muscidae flies (Jürgens et al. 2013), however, Muscidae flies do not appear to pollinate *S. hirsuta* var. *hirsuta*, therefore further studies

would need to address whether Calliphoridae and Sarcophagidae fly antennae respond to limonene with electroantennographic detection.

According to the ANOSIM analysis, *S. hirsuta* var. *hirsuta* appears to mimic dung, however the presence of sulphides in the headspace of these flowers suggest carrion mimicry is also present (Table 4, Table 6) (Jürgens et al. 2013; Jürgens & Shuttleworth 2015). Dimethyl disulphide was emitted in higher relative amounts than dimethyl trisulphide, and was emitted in very high relative amounts by the maroon morphs (Table 4). Dimethyl disulphide and dimethyl trisulphide are usually indicators of the early stages of carrion decay, as the volatile composition of carrion changes over time (Jürgens et al. 2013). Interestingly, dimethyl disulphide has also been reported as a fungal volatile and reportedly reduces oviposition by *Musca domestica* (Lam et al. 2010). However, dimethyl trisulphide has been reported to be emitted by dead vertebrates and a synergistic effect with dimethyl disulphide has been reported in the attraction of flies (Johnson & Jürgens 2010; Shuttleworth & Johnson 2010). This suggests that there is definitely carrion mimicry at play, however, the presence of *p*-cresol in the volatile emissions of these flowers suggest that there is also dung mimicry as *p*-cresol is strongly associated with dung (Schiestl & Dötterl 2012). It is likely that *S. hirsuta* var. *hirsuta* is not as specialised as some carrion and dung mimics (such as the dead-horse arum which very closely mimics the scent of dead gulls (Stensmyr et al. 2002)), but is rather generalist by mimicking the scent of both carrion and dung. Studies have also shown that there may be an interaction effect between scent and colour in the attraction of flies (Kugler 1957; Brodie et al. 2014; Woodcock et al. 2014).

Most studies have not examined odours and colours together and this study provides evidence that these may play a combined role. Interestingly, Kugler's (1957) studies on fly colour preferences showed that flies have a preference for yellow coloured models in the presence of sweet odours and a preference for darker coloured models in the presence of foul odours (such as dimethyl disulphide and dimethyl trisulphide). It appears that only the yellow flowers emit sweet scents in the form of limonene (Table 4). Furthermore, Zito et al. (2013) were able to show that sweet scents are also able to attract *Musca domestica* flies. Even though they did not specifically test limonene, it shows that sweet scents are attractive to some flies. Even though the human nose is unable to perceive the smell of the limonene emitted by the yellow flowers (still overpowered by foul odours), flies may be able to. This poses a very interesting question about the purpose of these different floral morphs in terms of colour and scent differences, and it should also be noted that the same assemblage of flies that visit the maroon morph also visit the yellow morph (Table 2). It could be due to an attempt to include a larger subset of pollinators, and without any trade-off, this may increase the likelihood of pollination by including a larger subset of morphologically similar pollinators. One would not expect a clear

trade-off as studies have shown that flies are indeed attracted to yellow coloured models (Burg & Axtell 1984; Woodcock et al. 2014), and to sweet scents (Zito et al. 2013) and the combination of sweet scents and yellow may be especially attractive to flies (Kugler 1957). Interestingly, these flowers occur in both yellow and dark coloured morphs.

A possible alternate explanation for the emergence of the colour morphs could be negative frequency-dependent selection. Gigord et al. (2001) showed that negative frequency-dependent selection maintains dramatic flower colour morphism in the rewardless *Dactylorhiza sambucina* (Orchidaceae). They suggested that rare colour morphs experienced a reproductive advantage as pollinators quickly learnt how to recognize and avoid (as the flowers are deceptive) the most frequent morphs. My results suggest that *S. hirsuta* flowers do not offer a nectar reward so a similar process may result in selection for different colour morphs in this species. However, it should be noted that many stapeliads do offer nectar concealed in a cavity below the guide rails and a microscope or dissection of the gynostegium may be required to absolutely confirm presence or absence of nectar (Bruyns 2005). In addition, *S. hirsuta* flowers appear to mimic fly oviposition sites and the resulting wastage of eggs/larvae may represent an additional cost, perhaps more important than the lack of a nectar reward. Although these was no evidence of oviposition on these flowers during this flowering season, flies oviposit extensively on cultivated *S. hirsuta* var. *tsomoensis* (pers. obs.), and the lack of oviposition could be due to a lack of gravid female flies during the current study period. Further studies would need to address the extent of fly oviposition on *S. hirsuta* var. *hirsuta*. These costs may alter the signal preferences of flies away from the common (in this instance the maroon morph) and result in the maintenance of the extreme floral morphism observed within this species. The possibility of negative-frequency dependent selection in *S. hirsuta* is supported by visitation rates of the most abundant visitors, Calliphoridae, to the different morphs (Table 2). The small numbers of Sarcophagidae and Muscidae flies observed visiting flowers may not be a large enough sample size for statistical significance, and further studies may be required to determine whether they have a floral morph preference. It is not clear, however, how these Calliphoridae flies are distinguishing the two morphs as my results suggest that flies perceive the colours of both morphs as fly-blue (Figure 9). One possibility is that there are achromatic differences which flies can perceive. Alternatively, flies may rely on the differences in odour between the two morphs (yellow flowers emitted higher relative amounts of limonene and lower relative amounts of *p*-cresol than maroon morph flowers; Table 2).

The role of trichomes on carrion-mimicking flowers has rarely been explored. This study has shown that the presence of trichomes has a positive effect on both the visitation rates as well as the amount of time that visitors spend probing flowers (Figure 11). This may well translate into increased

pollination success, although further studies could would be needed to confirm whether these differences might increase fitness. These results suggest that trichomes act as both a visual and a tactile cue (Figure 11). The floral trichomes likely allow the flowers to more closely resemble the surface of a decaying mammalian carcass, thereby representing a suitable oviposition or feeding site to flies (Jürgens et al. 2006; Moré et al. 2013; Urru et al. 2011; Raguso 2004). It is also possible that the subtle movement of trichomes in a breeze may aid in the attraction of flies (Vogel 1954). Bänziger (2001) has suggested that trichomes could also act as a tactile cue which stimulates oviposition in some blowflies, however, it is unclear how this would benefit the plant. It should also be noted that many stapeliads are in-fact glabrous, suggesting that hairy flowers are not a pre-requisite for the attraction and exploitation of carrion flies (Bruyns 2005). Geoflory could further aid in carrion or faecal mimicry as the position of carrion and faeces is usually at ground level (Johnson & Schiestl 2016).

Geoflory appears to have a significant impact on fly visits, as flowers lifted off the ground at 1 m received significantly less fly visits than flowers at heights of 0.5 m and lower (Figure 12). This is most likely, as Johnson & Schiestl (2016) suggested, due to the natural position of carrion as flies would not search for oviposition or feeding sites occurring outside of their natural position. Looking at bat-pollinated flowers could support this claim as many flowers that are bat pollinated do in fact emit sulphur containing compounds such as dimethyl disulphide and dimethyl trisulphide and are not visited by flies (Bestmann et al. 1997; Knudsen & Tollsten 1995; von Helversen et al. 2000). This is likely due to these flowers occurring in forest canopies high above the natural position of dung and carrion. However, not all carrion and dung mimicking flowers exhibit geoflory. Vines such as *Aristolochia grandiflora* (Aristolochiaceae) (Burgess et al. 2004) and *Ceropegia ampliata* (Asclepiadoideae) (Coombs et al. 2011) present their flowers well-above ground level and are both visited by carrion-associated flies. Some stapeliads, such as members of the genus *Hoodia*, also present their flowers well above ground level (up to c. 2m above the ground; Bruyns 2005). This implies that this strategy is not imperative in the attraction of flies, although future studies could explore whether particular fly types may be differentially sensitive to the height of suitable food or oviposition sites (and flowers).

This study examined two aspects of fly behaviour in the form of whether the flower effectively guides the fly to explore its gynostegium (necessary for effective removal and insertion of pollinia) and whether the fly lands directly onto the flower (suggesting a role for the flowers as a visual target for approaching flies). Jürgens et al. (2006) suggested that, in carrion mimicry systems, odour cues likely represent long distance attractants, while visual cues are more likely to be important for short distance

orientation to flowers. In general, flowers are thought to represent a visual target and landing platform for pollinators (Stebbins 1970). However, it is not clear how important these roles are in systems such as carrion mimicking flowers, which are thought to rely primarily on odours for pollinator attraction. I found that *S. hirsuta* flowers represent a suitable visual cue and landing platform as flies visited concealed flowers significantly less that visible flowers (Figure 10) and the majority of flies were found to land directly on flowers (Table 3). Wall & Fisher (2001) found in *L. sericata* that the flies are able to locate an odour source more efficiently when a visual cue is present. Concealing a flower had a negative impact on the number of visits compared to an exposed flower, which confirms the findings of Wall & Fisher (2001) and reinforces the importance of a visual cue for flower location by flies. Sarcophagidae were found to directly land on the flower 68.8% of the time, which was significantly different to the landing rates of Calliphoridae and Muscidae. This could imply that Calliphoridae and Muscidae rely more on visual cues in order to find the odour source. It is reasonable to assume that increased probing and exploring of the flower by the visitor would result in increased likelihood of pollination. My results provide evidence that the flies are guided very specifically to the gynostegium, as the majority of flies were found to move to the centre (Table 3). The exact mechanism the flower utilises to guide the flies to the gynostegium is unclear. One possibility is that flowers exhibit subtle odour gradients or have qualitative differences between different floral parts (Bergström et al. 1995; Dobson & Bergstrom, 2000; Knudsen & Tollsten, 1991). In future studies it would be interesting to explore mechanisms guiding flies to the centre of the flower.

Stapelia hirsuta experienced relatively high pollination success (30.2% of flowers were pollinated) at this site. Fruit set, however was lower (47.6 %), although still high compared to other stapeliads and asclepiads (Coombs et al. 2011; Shuttleworth & Johnson 2006; Shuttleworth & Johnson 2008; Shuttleworth & Johnson 2009a; Shuttleworth & Johnson 2009b). The difference between % of flowers pollinated and fruit set may be due to high levels of geitonogamy as stapeliads are likely to be self-compatible (Bruyns 2005; Meve et al. 2004). The reduced fruit set relative to insertion rates could also be due to resource limitation. Coombs (2010) reported that natural fruit set for *S. hirsuta* var. *baylissi* never exceeded 8% over three seasons and Shuttleworth et al. (2017) reported a natural fruit set of 13.7% for *O. lutea* subsp. *lutea*. In contrast Herrera and Nassar (2009) reported 35% fruitset for *Stapelia gigantea* in Venezuela where it is invasive. The pollen transfer efficiency of *S. hirsuta* var. *hirsuta* was 8.7% which is somewhat lower than that of other members of Asclepiadoideae which have been reported to in the range of 20-30% (Shuttleworth & Johnson 2012). Although the PTE of *S. hirsuta* var. *hirsuta* is still higher than that reported for *S. hirsuta* var. *baylissi* (3%) and *O. lutea* subsp. *lutea* (4.2%) (Coombs 2010; Shuttleworth et al. 2017).

Stapelia hirsuta var. *hirsuta* flowers are visited by a small subset of different carrion and faecal associated flies from three different families. This species is thus quite specialized although slightly more generalist than some other stapeliads such as *O. lutea* subsp. *lutea* and *S. hirsuta* var. *baylissi* (Coombs 2010; Shuttleworth et al. 2017). *Stapelia hirsuta* var. *hirsuta* is also rather generalist in terms of scent mimicry, as the scent profile of *S. hirsuta* var. *hirsuta* contains compounds associated with both carrion and dung mimicry. This can be considered generalist compared to the levels of specialisation of some other sapromyiophilous plants such as the dead-horse arum which closely mimics the scent emitted by dead gulls (Stensmyr et al. 2002). Interestingly, the yellow coloured morph also emits a higher relative amount of limonene (a sweet scent) than the maroon morph. Furthermore, the yellow morph also receives more visits from flies than the maroon morph, even though visually the colours of both morphs are indistinguishable by flies (Figure 9). Further studies would need to address whether limonene does elicit an electroantennographic response by carrion and faecal associated flies to determine if this compound is responsible for higher visitation rates. Further studies also need to address the possibility of negative-frequency dependent selection as well as comparing reproductive success of the two morphs to determine whether one has a selective advantage over the other. The presence of trichomes appears to be of importance in the attraction of flies and may also play a role as a visual and tactical cue. In this study, I have demonstrated that *S. hirsuta* is pollinated by carrion flies and I show a functional role for a visual cue, trichomes and geoflory in attracting flies and manipulating their behaviours, but many aspects such as the presence of nectar, reproductive success of different morphs and limonene as a fly attractant remain to be explored.

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CHAPTER 4

General discussion and conclusion

Stapeliads, and many other putatively carrion-mimicking plants, exhibit very unusual floral traits (Bruyns 2005; Jürgens & Shuttleworth 2015). Although these have often been assumed to enhance the resemblance of the flowers to carrion or faeces (the assumed models), empirical evidence for their role in manipulating pollinator behaviour remains scarce. In this thesis, I examined the pollination ecology of two stapeliads and explored the functional roles of unusual traits of *Orbea variegata* and *Stapelia hirsuta* var. *hirsuta* in the attraction of fly pollinators. This specifically included examining the roles of colours and patterning in the attraction of flies by *O. variegata*, the role of trichomes in the attraction of flies and any effects of flower positioning on fly visits by *S. hirsuta* var. *hirsuta*. Further, I also explored the importance of visual versus olfactory cues in both *O. variegata* and *S. hirsuta*. In addition, I included detailed observations on the natural history and the ecology of these flowers and quantitative analyses of certain floral traits. The intention of this chapter is to summarise and discuss the major findings on the functional significance of the floral traits of *O. variegata* and *S. hirsuta* var. *hirsuta*, and discuss these roles in the context of carrion and dung mimicry in general.

POLLINATION AND REPRODUCTIVE BIOLOGY

Many stapeliads are believed to be pollinated by carrion and dung associated flies (Bruyns 2005; Meve & Liede 1994). In this study, I have shown that *Orbea variegata* flowers are pollinated by members of Calliphoridae, Muscidae and Sarcophagidae (Chapter 2, Table 3), whereas *Stapelia hirsuta* var. *hirsuta* flowers are pollinated by Calliphoridae and Sarcophagidae (Chapter 3, Table 3). Interestingly, *S. hirsuta* var. *hirsuta* flowers are also visited by flies belonging to Muscidae, although they do not appear to be pollinators. Meve & Liede (1994) have suggested that the size relation between the mouthparts of flies and the guide rails of stapeliads needs to be appropriate for flies to be able to pick up pollinia; it is likely that the size of the mouthparts of Muscidae flies is not suitable for pollinium attachment and they are therefore unable to act as pollinators for *S. hirsuta* var. *hirsuta*. The size of these guide rails may lead to specialisation to certain flies in the form of a morphological filter, as is the case with *O. lutea* subsp. *lutea* where the spacing between the inner corona lobes only allows for certain sized flies to fit through (Shuttleworth et al. 2017). *Orbea lutea* subsp. *lutea* is only

pollinated by a single species of *Atherigona* (Muscidae), as these are the only ones that could fit between the inner corona lobes, which is necessary to reach the guide rails (Shuttleworth et al. 2017).

Both *O. variegata* and *S. hirsuta* var. *hirsuta* have abnormally high fruit set compared to that of other stapeliads (Coombs 2010; Harder & Johnson 2008; Shuttleworth et al. 2017; Herrera & Nassar 2009) and other milkweeds (Shuttleworth & Johnson 2006; Shuttleworth & Johnson 2008; Shuttleworth & Johnson 2009b; Shuttleworth & Johnson 2009a) (Chapter 2, Chapter 3). The pollen transfer efficiencies (PTE) for *O. variegata* and *S. hirsuta* var. *hirsuta* were slightly lower than those reported for other members of Asclepiadoidae (Shuttleworth et al. 2017), but still higher than the PTE reported for *O. lutea* subsp. *lutea* (Shuttleworth et al. 2017) and *S. hirsuta* var. *baylissi* (Coombs 2010) (Chapter 2, Chapter 3).

VISUAL SIGNALLING

Visual signals exhibited by flowers are believed to play an important role in the attraction of pollinators (Schiestl & Johnson 2013), and interestingly, the floral colours and patterning of carrion and dung associated flowers are convergent across many plant lineages (Jürgens et al. 2006; Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015). The evolution of the sensory modalities and associated preferences of carrion and dung associated flies (and detritus-feeding insects in general) is believed to be linked to the visual and olfactory cues used by flies to locate carrion and dung (Jürgens & Shuttleworth 2015). Carrion and dung mimicking flowers rely on these insects' innate response to food sources or oviposition sites (Jürgens & Shuttleworth 2015). In terms of visual signalling, plants associated with carrion or faecal mimicry are often brown and dark red (such as *S. hirsuta* var. *hirsuta*) or yellow (such as *O. variegata* and *O. lutea*) and sometimes even black in colour (Bruyns 2005; Johnson & Jürgens 2010; Jürgens & Shuttleworth 2015; Moré et al. 2013). These deep red colours have often been suggested to aid in the mimicry of a decomposing carcass (Chen et al. 2015; Johnson & Schiestl 2016), in addition to this, studies have shown that flies prefer black (or dark coloured models) in the presence of foul odours (Brodie et al. 2014; Kugler 1957). Chapter 2 provides evidence that flies prefer black flowers to yellow flowers in the presence of *O. variegata* odour. This result is unexpected as *O. variegata* flowers are predominantly yellow in colour (Figure 1, Chapter 1). *Orbea variegata* is not the only plant believed to be a carrion or faecal mimicking with bright yellow colouring, many other stapeliads also contain yellow colouring (such as *O. lutea* ;Bruyns 2005; Shuttleworth et al. 2017), as well as members of the genera *Ferraria* (Iridaceae) (Goldblatt et al. 2009), *Periploca* (Asclepiadoidae) (Pisciotta et al. 2011) and many more. It is also possible that the

yellow colouring serves a purpose unrelated to pollination. One possible explanation for the yellow colouring in *O. variegata* could be thermal management, as these flowers generally occur in full sunlight (pers. obs.). McKee & Richards (1998) showed in their study on *Crocus chysanthus* and *C. tommasinianus* that yellow coloured flowers exhibit the least warming compared to white and purple flowers. Further studies would need to address possible explanations for the yellow colouring.

Floral patterning is common on stapeliads (Bruyns 2005; pers. obs.) and has also been suggested to play an important role in the attraction of flies (Johnson & Schiestl 2016; Jürgens et al. 2006; Jürgens & Shuttleworth 2015). However, this does not appear to apply to *O. variegata*. No empirical studies have examined the potential role of blotching on carrion or faecal mimicking flowers, however, Chapter 2 provides evidence that the presence as well as the size of the blotching has no significant effect on fly visits. The size of blotching on the corolla lobes varies greatly on *O. variegata* (Chapter 2, Figure 2), so it is possible that this specific form of patterning could be selected for by factors not relating to pollination of the flower. Possibilities could range from serving to break up the outline of the flower or resemblance to shadows to conceal flowers, although this would need to be addressed in further studies. Futhermore, my results suggest that flies cannot perceive a chromatic difference in the colouring of the blotching and the yellow corolla lobes, as both are perceived as fly-green (Chapter 2, Figure 6). Further studies could examine a possible achromatic contrast between the blotching and corolla lobes in terms of fly vision. Sutherland et al. (1999) showed that the hoverfly *Episyrphus balteatus* can distinguish between similarly coloured yellow artificial flowers, which suggests that some flies do have the ability to distinguish achromatic contrasts. Futhermore, bees have been shown to perceive contrasts within green, and this contrast is relevant because bees use only the green receptors for certain visually guided tasks (Giurfa et al. 1995), therefore it is possible that other insects could also perceive achromatic contrast. The deep red and brown colouring and patterning often found on sapromyiophilous flowers is believed to assist in the attraction of carrion associated insects, as these colours can be associated with a decaying animal carcass (Bänziger 2001; Chen et al. 2015; Johnson & Schiestl 2016). The visual mimicry in sapromyiophilous plants can be rather extreme; for example, the appearance of the dead-horse arum can be likened to the rump of a dead animal, and resemblances can be noted for even an anus and a tail (Johnson & Schiestl 2016; Seymour et al. 2003).

Another visual (or tactile) trait commonly associated with carrion and faecal mimicry is the presence of trichomes on the flowers (Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015). The floral trichomes of *S. hirsuta* var. *hirsuta* are unicellular hairs, where density and thickness can vary greatly (Bruyns 2000; Bruyns 2005; pers. obs.). Floral trichomes differ from papillae in that papillae are fine

(uni- or multicellular) hairs found on the stems and outsides of flowers of stapeliads as well as raised ridges (which are fused papillae) and tubular growths (e.g. on *Huernia hystrix*) (Bruyns 2005). Papillae are present on the stems and outsides of flowers of members belonging to *Stapelia* and are unicellular (Bruyns 2005). Furthermore, clavate cilia are also present on some stapeliads, such as *O. luteta*, where they are vibratile or loosely pendulous on the corolla lobes (Bruyns 2005; Meve & Liede 1994). However, clavate cilia are not present on *S. hirsuta* var. *hirsuta* flowers. The functional significance of the trichomes often found on sapromyiophilous plants has not been studied, however suggestions concerning their role on the flowers have been made. One of these suggestions is that the trichomes (as a visual cue) allow these flowers to mimic a dead animal carcass more closely (Jürgens et al. 2006; Moré et al. 2013; Raguso 2004; Urru et al. 2011). As a tactile cue, trichomes have been suggested to induce oviposition in some blowflies; however, it is unclear how this would benefit a flower (Bänziger 2001). Therefore, the exact function that floral trichomes serve is not clear, although the functional significance of these trichomes has, in this study, been shown to be of importance in the attraction of flies in *S. hirsuta* var. *hirsuta*. The presence of trichomes on *S. hirsuta* var. *hirsuta* flowers significantly increased fly visits, and therefore clearly acts as a visual signal for these flowers (Chapter 3, Figure 11). It is also possible that floral trichomes act as a tactile cue, this study provides evidence that in *S. hirsuta* var. *hirsuta*, trichomes play an important role as a tactile cue, as their presence results in flies staying on flowers for longer periods of time (Chapter 3, Figure 11). Further studies could address whether trichome presence translates into a reproductive advantage (such as pollen transfer efficiency).

FLORAL ODOURS

Carrion and faecal mimicking flowers are well-known for their putrid scents which have usually been assumed to play a role in the attraction of carrion and dung associated insects for pollination (Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015; Jürgens et al. 2013). Although studies explicitly testing the role of these odours are scarce, detailed analyses of the chemistry of some of these odours by Jürgens et al. (2006) suggested that these flowers mimic different substrates (such as dung, carrion or urine) as the compounds emitted by these flowers can be associated with volatiles produced by these substrates.

Carrion and faecal mimicking flowers are also known mimic the scent profiles of their pollinator's oviposition or feeding sites with striking accuracy, for example, Stensmyr et al. (2002) found that the dead-horse arum almost exactly mimics the scent of a dead gull. Since these odours emitted by carrion

and faecal mimicking plants are so essential to the detritus-feeding insects, the insects cannot afford to ignore the signal (Stensmyr et al. 2002). The odours of *O. variegata* and *S. hirsuta* var. *hirsuta*, which likely serve as the long distance attractants (Jürgens et al. 2006), was shown in both species to be characterised by dimethyl trisulphide amongst other compounds. Dimethyl trisulphide appears to be an important compound in the olfactory signalling of many carrion and faecal mimicking flowers (Jürgens et al. 2013; Moré et al. 2013; Shirasu et al. 2010; Stensmyr et al. 2002). This likely relates to the prevalence of dimethyl disulphide and dimethyl trisulphide in the degradation odours of protein and therefore carrion odour (Jürgens & Shuttleworth 2015; Jürgens et al. 2013). Even though other oligosulphides (such as dimethyl disulphide) are often produced in addition to dimethyl trisulphide as floral volatiles, Zito et al. (2013) have shown that dimethyl trisulphide is likely to be the key compound in the attraction of members of Calliphoridae and Muscidae as opposed to dimethyl disulphide. Even though dimethyl disulphide was present in the scent profile of both *O. variegata* (very low amounts and not present in every sample) (Chapter 2, Table 6) and *S. hirsuta* var. *hirsuta* (Chapter 3, Table 6), dimethyl trisulphide was shown to not only be present in each sample, but also highly characteristic of the scent profile of both plants. Interestingly, even though the scent profiles of *O. variegata* and *S. hirsuta* var. *hirsuta* are both characterised by oligosulphides (which are normally associated with carrion mimicry and decaying proteins (Jürgens et al. 2013)) (Chapter 2, Table 7; Chapter 3, Table 7), the ANOSIM analyses suggest that both are dung mimics rather than carrion mimics (Chapter 2, Table 8; Chapter 3, Table 8). However, with the presence of dung and carrion associated compounds in the scent emitted by both flowers, it is likely that they mimic both carrion and dung. There is likely no trade-off between emitting compounds associated with carrion and faecal volatile emissions, as flies attracted to carrion may be morphologically similar to the flies that feed on faeces, therefore are functionally similar as pollen vectors (Johnson & Schiestl 2016). Further studies could use electroantennographic detection to determine the active compounds for flies found to pollinate these flowers. Flies could also be collected from carrion and dung, and compared to the assemblages pollinating these flowers to determine whether the pollinators are associated with carrion or dung.

COMBINED EFFECTS OF VISUAL AND OLFACTORY CUES

The visual and olfactory signals of any potential feeding or oviposition sites are known to vary greatly, which requires the flies to evolve a very fine-tuned olfactory system, in order to find and determine the quality and quantity of the food source (Jürgens & Shuttleworth 2015). Empirical studies have shown that carrion and dung associated flies show a preference for dark models in the presence of

carrion or faecal scents and for yellow (or light coloured models) in the presence of sweet scents (Brodie et al. 2014; Kugler 1957; Woodcock et al. 2014). The reason why many of these sapromyiophilous flowers are yellow (or contain yellow) is not well-understood. Fly preference for yellow in the presence of sweet scents suggests that there is an innate preference for the colour yellow, however, there may also be non-pollination related evolutionary influences for the selection of yellow coloured flowers. Interestingly, *S. hirsuta* var. *hirsuta* did emit sweet-scented compounds (limonene), but only the yellow coloured morph, whereas, with the exception of a single flower, the maroon morph did not emit this compound (Chapter 3, Table 6). Furthermore, the rarer yellow morph was also visited more by Calliphoridae than the maroon morph (Chapter 3, Table 4), and it is believed that polymorphism in deceptive plants is due to negative-frequency dependent selection (Gigord et al. 2001). Gigord et al. (2001) suggested that rare coloured morphs would have a reproductive advantage over abundant colours morphs when no reward is offered. Further, the importance of visual cues in addition to olfactory cues is evident when comparing fly visits to a visible flower to fly visits to a concealed flower. My studies showed that both *O. variegata* and *S. hirsuta* benefit from the presence of a visual cue, as visible flowers received significantly more fly visits than concealed flowers.

FLOWER POSITIONING

Geoflory is another feature that is strongly associated with carrion and faecal mimicking flowers, which is believed to be due to the natural position of the carrion or faecal models they are mimicking (Johnson & Schiestl 2016; Jürgens & Shuttleworth 2015; Vogel & Martens 2000). Geoflory is a very common feature in the stapeliads (although a few do not exhibit geoflory, such as *Hoodia* where flowers are located meters off the ground; Bruyns 2005), and particularly *O. variegata* and *S. hirsuta* var. *hirsuta* flowers are always located at ground level (Bruyns 2005; pers. obs.). Other examples of sapromyiophilous plants that exhibit geoflory include the dead-horse arum (Stensmyr et al. 2002) and the South African orchid *Satyrium pumilim* (van der Niet et al. 2011). Again, not all sapromyiophilous plants exhibit geoflory, one exception being members of the genus *Aristolochia* (Jürgens & Shuttleworth 2015). Notably, many bat pollinated flowers situated high in the forest canopy also emit sulphides but are not visited by flies (Bestmann et al. 1997; Knudsen & Tollsten 1995; von Helversen et al. 2000). This is likely due to the elevated location of these flowers. My results suggest that geoflory does play a significant role in the attraction of flies in *S. hirsuta* var. *hirsuta*, as there was a significant impact on fly visits when flowers are lifted off the ground, where flowers situated higher than 0.5 m off the ground received less visits than flowers at 0.5 m and lower (Chapter 3, Figure 12).

CONCLUSION

This study suggests that floral signalling is key for the attraction of pollinators, and that there is likely an interactive effect between the visual and olfactory cues in the attraction of insects. In terms of visual versus olfactory signalling, they are likely to go hand in hand, and it has been suggested neither would be successful without the other (Pellmyr & Thien 1986; Wall & Fisher 2001). The presence of trichomes as a visual and tactile cue as well as the position of the flower has shown to be functionally significant. The functional significance of floral patterning remains a mystery as it has no effect on fly visits. This study further supports black as a preferred colour in the presence of putrid scents (Brodie et al. 2014; Kugler 1957), however, the rarer *S. hirsuta* var. *hirsuta* yellow morph received more fly visits than the (darker) maroon morph. In contrast, Chapter 2 shows flies prefer dark models, therefore, this higher visits to the yellow morph could be due the presence of limonene in the floral scent bouquet or negative frequency-dependent selection. Further studies would need to address this question.

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