Behavioural responses of rodents to the scent and taste of compounds associated with sugar and protein degradation: implications for the evolution of chemical signals in rodent-pollinated flowers

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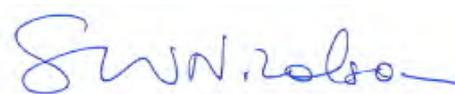
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Abstract. Volatile compounds in nectar may influence the behavioural responses of animal flower visitors, and thus have fitness consequences for both animal and plant mutualists. Rodents may use certain volatiles associated with sugar fermentation or protein degradation as a cue to locate food. Plants pollinated by rodents may thus emit these volatiles to enhance their attractiveness to rodents. However the presence of certain compounds in nectar may also indicate reward degradation, reducing its attractiveness to potential pollinators. The effects of these compounds on small mammal flower visitors are largely unknown and the consequences of nectar degradation by microorganisms for small mammal flower visitors need investigation. The present study examines the responses of a known rodent pollinator, the Namaqualand rock mouse *Micaelamys namaquensis*, an occasional floral visitor, the four-striped field mouse *Rhabdomys pumilio*, and a closely-related congener, the mesic four-striped field mouse *Rhabdomys dilectus* towards four compounds - ethanol, ethyl acetate, acetic acid and dimethyl disulphide - that are associated with the degradation of sugars and proteins. The study aimed to: (i) Identify if fermentation and protein degradation volatiles act as behavioural cues for small mammals, and have the potential to assist in the finding of food resources; and (ii) to determine the responses of rodents to the taste of volatiles in nectar.

In chapter 1, I investigated whether fermentation and protein degradation volatiles elicit a behavioural response in small mammals, using a traditional Y-maze choice apparatus. Rodent species differed in their responses to the four volatiles tested. Ethanol emerged as an attractant for all of the species, with the strongest response seen at the 0.3 % ethanol concentration, while only *R. dilectus* responded positively to dimethyl disulphide. Acetic acid and ethyl acetate were generally not attractive.

In chapter 2, the palatability of fermentation and protein degradation compounds at varying concentrations in experimental nectars was tested. A paired choice test analysis was used to determine if animals altered their choice with the addition of volatiles to sucrose solutions (0.73 and 1.46 M). Rodents showed a dose-dependent response towards the volatile concentrations presented to them. Both *M. namaquensis* and *R. pumilio* preferred medium (0.3 %) concentrations of ethanol in high sugar concentration diets, but found this compound distasteful in lower sugar concentration diets. Acetic acid and ethyl acetate were generally not preferred by any species at either sugar concentration. Dimethyl disulphide was preferred by all species when present in greater sugar concentrations (1.46 M) and at low volatile concentrations (0.003 %). These results suggest that rodents do alter their choice of nectar in relation to the tested compounds.

I conclude that certain compounds associated with sugar fermentation or protein degradation have the potential to act as behavioural cues in rodent pollination and that further studies to reveal the effect of nectar degradation by microorganisms are needed to help in understanding plant-pollinator interactions.

Key words: Microorganisms, Ethanol, Dimethyl disulphide, Pollination, Behavioural response

Chapter 1: General Introduction

The importance of pollinators in the diversification and radiation of angiosperms was first addressed by Darwin (1862; Friedman 2009). Subsequent explanations however focused on the contribution of abiotic factors (eg rainfall, topography, soils and fire regimes) to angiosperm diversity and distributions (Goldblatt 1978; Linder 2003). From the late twentieth century to the present there has been a revival of interest in the importance of plant-pollinator interactions for the angiosperm radiation (Grant and Grant 1965; Johnson 1996; Raguso 2004; Johnson 2010). This has been sparked by compelling evidence for the role of adaptation to pollinators in shaping floral divergence among species. A classic example is the evolution of dull-coloured geoflorous yeasty-scented inflorescences in many different ancestrally bird-pollinated clades of Proteaceae in the Cape mountain region (Johnson 2006). These flowers are pollinated by small rodents (Wiens and Rourke 1978). Is this an example of pollinator driven speciation through the radiation of floral characteristics? If so, could the shift from bird to rodent pollinators in these lineages have initially been promoted by attraction of rodents to the smell of fermenting nectar?

Pollination syndromes

When a set of unrelated plants undergoes floral adaption to a particular suite of pollinators, a common outcome is patterns of convergent floral evolution (pollination syndromes) among these plants. These syndromes reflect the sensory preferences, physiology and morphology of pollinators (Faegri and van der Pijl 1971; Rourke and Wiens 1977). Syndromes encompass a wide range of floral traits including shape, size, colour, floral anthesis and odour, and rewards such as nectar and pollen. For example, the floral syndrome associated with beetle pollination is associated with robust, bowl shaped flowers with strong fruity scent, large pollen loads and copious amounts of nectar (Bernhardt 2000; Shuttleworth and Johnson 2010; Steenhuisen and Johnson 2012), whereas that associated with rodent pollination involves dull coloured robust flowers, situated at ground level, coupled with strong yeast-like odours and nocturnal floral anthesis (Wiens et al. 1983; Johnson et al. 2001; 2011). Further still, the floral syndrome associated with bird pollination involves colourful, often red, flowers, which produce very dilute nectar (Stiles 1978; Rebelo et al. 1984; Manning 2009). Syndromes themselves may be subdivided in a hierarchical fashion,

an example being flowers pollinated by sunbirds and hummingbirds which have more concentrated nectar, richer in sucrose, than nectar in flowers pollinated by opportunistic generalist birds (Johnson and Nicolson 2008).

To understand the evolution of floral syndromes in animal-pollinated plants, it is necessary to investigate the underlying causal factors involved in selection by animals. Sensory differences among animals may account for the emission of particular volatiles by a plant or its nectar (Pichersky and Gershenzon 2002; Raguso 2004; Wright and Schiestl 2009; Schiestl 2010). Taste preferences could explain the evolution of the sugar composition of nectar or secondary compounds that are present in nectar (Omura and Honda 2003; Irwin et al. 2004; Irwin and Adler 2008). Body size and activity patterns of animals may be linked with the amount and type and timing of secretion of rewards offered by a plant (van Tets 1997; Fleming and Nicolson 2002; Davies et al. 2003).

Almost all animal pollinators have acute olfactory senses that assist in finding mates, foraging and defending territories. Therefore volatiles emitted by plants, often in combination with visual cues, function to enhance pollinator attraction over distances and ensure fitness in plant-pollinator interactions (Pichersky and Gershenzon 2002; Raguso 2008; Wright and Schiestl 2009). The use of scent could be an invaluable cue for pollinators in night active pollination systems (Raguso 2004; Johnson et al. 2011). For example, bat pollination often relies on flowers which utilize strong sulphur containing fragrances to attract pollinators when visual cues would be less effective (von Helversen et al. 2000). Another example is that of plants pollinated by moths, such as *Silene* species (Caryophyllaceae), whose floral volatiles include linalool and benzyl alcohol, which are released during nocturnal floral anthesis when pollinators are most active (Ellis and Ellis-Adam 1993; Jürgens et al. 2002; Young 2002).

Taste is another floral cue utilized by floral visitors and can be a decisive factor in flower selection (Baker and Baker 1982; 1983; Nicolson and van Wyk 1998; Johnson et al. 2006; Zhang et al. 2012). Critical nectar components related to floral selection include the type, relative contributions and concentrations of sugars, all of which dictate how long a visitor will remain at a particular plant before moving on in search of its next floral reward (Baker and Baker 1983). However in certain cases other compounds may be used to ensure frequent floral visitation (Baker and Baker 1982).

For example, amino acids in nectar may contribute to pollinator attraction and feeding in female cabbage white butterflies (*Pieris rapae*) and honey bees (*Apis mellifera*) (Alm 1990).

Additionally floral visitation by pollinators could be due to the presence of alternate resources offered by flowers. These include pollen, waxes, resins and oils, all of which have been documented as utilized during pollinator visitation (van Tets 1997; Johnson et al. 1999; Davies et al. 2003; Irwin et al. 2004; Singer and Koehler 2004). Rodents, for example, show increased visitation and utilization of pollen during the later winter periods when certain Proteaceae species commence flowering (Wiens et al. 1983; Fleming and Nicolson 2002; Turner et al. 2011). Stingless bees, the main pollinator for the genus *Maxillaria* (Roubik 2000), collect wax and resin like secretions from multicellular trichomes which are used as food for larvae or as nest-building material (Singer and Koehler 2004). Secretion of oils by flowers of some Orchidaceae has facilitated pollination by oil-collecting bees in South Africa (Steiner 1989; Johnson 1992).

Mammal pollination

Mammal pollination was first discussed over 80 years ago by Porsch (1934). However substantial evidence for pollination by nonflying mammals was only gathered 50 years later, through studies of South African protea species (Wiens and Rourke 1978). This discovery stimulated further research into mammal pollination and the recognition of distinct systems of bat and non-flying mammal pollination (Wiens et al. 1983; Carthew and Goldingay 1997).

Bat pollination or chiropterophily is characterised by dark red to creamy pink flowers with strong sulphur containing fragrances released during nocturnal floral anthesis when pollinators are most active (Harris and Baker 1958; Bestmann et al. 1997; von Helversen et al. 2000). Flowers are often large, bell shaped and produce copious amounts of dilute nectar (Faegri and van der Pijl 1971; Baker and Baker 1983). In Africa there have been studies of bat pollination in trees *Kigelia africana* and *Adansonia digitata* (Harris and Baker 1958), but chiropterophily is more widespread among neotropical plants (Hopkins 1984; Fleming and Muchhala 2008).

Non-flying mammal pollination or therophily is characterised by dull coloured flowers, geoflorous in nature, coupled with strong "pungent" or "yeasty" aromas associated with nocturnal floral anthesis (Wiens and Rourke 1978; Wiens et al. 1983; Carthew and Goldingay 1997). Non-flying mammal pollination was initially described in Australian Proteaceae species and has since been documented in the proteas of the Western Cape of South Africa and other plant families, including *Leucospermum arenarium* and *Protea foliosa* (Proteaceae; Johnson and Pauw 2014; Melidonis and Peter 2015), *Massonia depressa* (Hyacinthaceae; Johnson et al. 2001) and parasitic plants (Cytinaceae; Johnson et al. 2011; Hobbhahn and Johnson 2013). Other examples of plants attracting rodents are *Cajophora coronata* from Argentina and *Nepenthes rajah* from Borneo (Cocucci and Sersic 1998; Greenwood et al. 2011; Wells et al. 2011).

Sugar and protein degradation in nectar

Pollination systems have the potential to be altered by a third party interaction (de Vega et al. 2009). These third party players include microorganisms such as nectar-inhabiting yeasts and bacteria, both of which have been found to modify nectar composition in terms of sugar proportions and overall concentrations (Herrera et al. 2008; 2009; Vannette et al. 2012). These nectar-inhabiting microorganisms may weaken plant-pollinator mutualisms by decreasing floral attractiveness through reduced rewards for pollinators, thereby resulting in a decrease in fitness for plants and their pollinators (Raguso 2004; Herrera et al. 2008). The density of microorganisms in nectar appears to be related to the type of pollinator, with larger pollinators being associated with greater microorganism densities in their nectars (de Vega et al. 2009). The presence of microorganisms in the nectars of various plant species has stimulated interest among pollination biologists in finding out if the associated volatile compounds have the potential to alter plant-pollinator interactions (Herrera et al. 2008; Vannette et al. 2012; Records 2012).

Nectar characteristics altered through the presence of third party players in nectar include not only a reduction of sugars in floral nectars but also the production of volatiles indicative of sugar degradation - such as ethanol, acetic acid, and ethyl

acetate - under aerobic conditions (Janzen 1977; Goodrich et al. 2006; Madigan and Martinko 2006). These volatiles indicative of "nectar degradation" have been repeatedly observed in the nectars consumed by insects (Chakir et al. 1993; Milan et al. 2012; Omura and Honda 2003); as well as in vertebrate pollination systems, such as birds (Eriksson and Nummi 1982; Prinzinger and Hakimi 1996; Mazeh et al. 2008; Steenhuisen 2012; Records 2012); bats (Sanchez et al. 2004, 2006, and 2008) and non flying mammals (Wiens et al. 2008). The effect of fermentative volatiles in the nectars of various pollination systems needs investigation to determine if these volatiles either deter or attract pollinators.

Certain volatiles released by the food of rodents have been documented in seed detection, where rodents use the volatile emissions from seeds as scent cues to aid in seed locating (Hollander et al. 2012; Paulsen et al. 2013). Desert hamsters (*Phodopus roborovskii*) were found to use seed volatiles – likely to be ethanol, acetic acid and sulphur compounds – to effectively locate buried seeds under laboratory conditions (Paulsen et al. 2013). Volatile compounds associated with sugar and protein degradation have also been found in the floral bouquet of plants which are pollinated by rodents (Balmer 2013). These volatiles may be the reason that flowers of rodent-pollinated species have been described as being perceived by the human nose as "yeasty" or "pungent" smelling (Wiens et al. 1983; Johnson et al. 2001). This has led to interest in identifying if volatiles associated with sugar and protein degradation of nectar are indicative of diminished rewards for rodent pollinators, or if they are selected for in rodent pollination as a floral cue. Could volatile compounds indicative of fermentation be used as a cue in nectar selection by rodents? If plants could emit these compounds from floral parts and thus increase the signal of sugar and protein degradation, would this increase the rate of rodent visitation? If these compounds were present in nectar, would rodents be attracted or repelled by the taste?

The effects of these compounds on small mammal flower visitors are largely unknown and the influence of nectar degradation by microorganisms on small mammal flower visitors needs investigation. I investigated the behavioural effect of three volatiles indicative of nectar degradation - ethanol, ethyl acetate, acetic acid - and one indicative of protein degradation - dimethyl disulphide - on three rodent species: a well-known pollinator, the Namaqualand rock mouse *Micaelamys namaquensis* (Wiens and Rourke 1978; Wiens et al. 1983), an occasional floral

visitor, the four-striped field mouse *Rhabdomys pumilio* (Rourke and Wiens 1977; Wiens et al. 1983) and a closely-related congener, the mesic four-striped field mouse *Rhabdomys dilectus,* which is not a well known floral visitor (Johnson et al. 2011).

Rodent study species

Micaelamys namaquensis

The Namaqualand rock mouse *M. namaquensis* is distributed throughout southern Africa except the Namib Desert (Nowak 1999) and is commonly found in open woodland and rocky outcrops (Skinner and Chimimba 2005). Body mass of the animals used in these experiments was around 57 g (males, n = 7, 56.1 ± 3.7 g; females, n = 6, 58.8 ± 5.4 g). Individuals are nocturnal in habit, with activity periods peaking between 19h00 and 05h00 (Wiens et al. 1983). They feed predominantly on grains, but are known visitors of *Protea* species, having been recorded foraging on flowering heads of *Protea* species in the Cape (Wiens et al. 1983; van Tets 1997; Nowak 1999).

Rhabdomys pumilio

This taxon, the four-striped field mouse, is geographically limited to the western part of South Africa (Roberts 1951; Nowak 1999; Skinner and Chimimba 2005). Mass of the animals used in the study differed between sexes (males, n = 4, 43.4 ± 4.4 g; females, n = 3, 27.4 ± 1.5 g). Animals are crepuscular, with periods of high activity in early morning (06h00 - 08h00) and evening (16h00 - 18h00) (Wiens et al. 1983; Skinner and Chimimba 2005). They are generalist omnivores, feeding on the nectar and pollen of flowers (Rourke and Wiens 1977), vegetable matter, sometimes seeds and insects (De Graaff 1981; Nowak 1999). They have been reported as floral visitors in several studies (Wiens et al. 1983; Hobbhahn and Johnson 2013; Johnson and Pauw 2014; Melidonis and Peter 2015).

Rhabdomys dilectus

The second taxon of *Rhabdomys* used in experiments was the mesic four-striped field mouse *R. dilectus,* which is considered distinct from *Rhabdomys pumilio* on the basis of molecular evidence (Rambau et al. 2003). *R. dilectus* inhabits the moist eastern grasslands of South Africa, although its distribution tends to be patchy and discontinuous (Nowak 1999; Skinner and Chimimba 2005; Schradin and Pillay 2005). The body mass of the individuals used in the study differed between sexes (males, n = 5, 49.2 ± 4.1 g; females, n = 4, 42.3 ± 1.9 g). Animals in the wild are generally diurnal, however due to the grass habitat structure they have been found to be active throughout the day, as cover in the moist grassland biome can allow for all day foraging (Mackay 2011). They are generalist omnivores feeding on nectar from flowers, seeds, fallen fruits and insects (De Graaff 1981; Nowak 1999; Johnson et al. 2011).

Study sites

Ukulinga

Rhabdomys dilectus was trapped at Ukulinga, a farm belonging to the University of KwaZulu-Natal Pietermaritzburg (27^0 67' 12" S, 30^0 40' 27" E, elevation 680 m). The grass field in which the study took place is a savanna type ecozone scattered with trees (*Acacia karroo*, *A. nilotica* and *A. sieberiana*) and is located on a south facing slope (pers. observation). Rodent traps were placed within the study site at locations along a fence line and were spaced at 5 m intervals. Sherman traps (80 x 90 x 230 mm) were used for capturing rodents. Forty traps were laid out in two locations along the fence lines within the area, and left for a 24 hour period from mid-morning till mid-morning. The traps were cleared out if a rodent was caught, re-baited and then re-set in order to accumulate an adequate sample size. A mixture of rolled oats and peanut butter was the chosen bait.

Houw Hoek and Sir Lowry's Pass

Rodent trapping in the Western Cape was carried out to acquire individuals of *Micaelamys namaquensis* and *Rhabdomys pumilio* needed for experiments. Two sites were identified for trapping, Houw Hoek pass (34^0 12' 49" S, 19^0 10' 02" E; elevation, 340 m) and Sir Lowry's pass (34^0 08' 59" S, 18^0 55' 42" E; elevation, 480 m). Rodent traps were laid out between the rocky outcrops that were distributed throughout these passes. Sherman traps were spaced apart at 5 m intervals. Forty traps were laid out in two locations at each pass, and left for a 24 hour period from mid-morning till mid-morning. Traps were baited with rolled oats and peanut butter, and nesting material, grass twigs and leaves, was added to the traps for safety of the rodents. The traps were cleared out if a rodent was caught, re-baited and then re-set in order to accumulate an adequate sample size.

Rodent maintenance

All animals were transported under permit (permit number: 0011-AAA007-00670) and were housed in an animal house facility in a constant environment room with the temperature maintained at 25 ºC ± 1 ºC and a photoperiod of 14 L: 10 D. Rodents were individually housed in 30 × 30 × 50 cm glass aquaria with galvanized steel grid lids (Fig. 1). Maintenance diets were made up of a combination of seed mix, commercially available rodent pellets (AVI products, Kloof, South Africa), grated seasonal fruits and vegetables, and a combination of larval and adult mealworms. All diets were portioned and unique for each species of rodent depending on diet preferences in the wild and in relation to the weight of individuals. Food was provided in a stainless steel bowl placed in the cage every afternoon to maintain natural behavioural cycles in the animals. Water was provided *ad libitum* in a stainless steel bowl, cleaned and replaced daily. Liquid activated wood pellets were provided as bedding and nesting material (AVI products, Kloof, South Africa). Shredded paper, egg cartons and cardboard rolls were offered bimonthly for enrichment. Rodent enclosures were set up with wooden dowels or tree branches to encourage exploration and ensure well-being of animals.

Figure 1: Glass aquaria with galvanized steel grid lids where rodents were individually housed (30 × 30 × 50 cm). Enclosures were set up with tree branches to encourage exploration and allow for gnawing. Liquid activated wood pellets were provided as bedding and nesting material included shredded paper and egg cartons. Water was provided *ad libitum* in a stainless steel bowl.

Thesis outline: specific research questions and aims

This thesis is presented in two chapters that cover two different research questions related to the responses of rodents to compounds arising from sugar and protein degradation. These questions are:

1. Could the volatiles arising from sugar and protein degradation be used as behavioural cues by small non-flying mammals?

2. Do these volatile compounds in nectar influence the palatability of nectar to rodents, leading either to increased or decreased consumption?

Chapter 2: **Behavioural responses of rodents to the scent of volatile signals arising from sugar and protein degradation.**

Plants visited by rodents are described as having "pungent" and "yeasty" aromas, released during nocturnal floral anthesis, hinting at the potential for the volatiles to be used as behavioural cues by small mammal pollinators in locating nectar or pollen rewards (Wiens and Rourke 1978; Wiens et al. 1983; Johnson et al. 2001). The attraction of rodents to volatiles associated with fermentation is not well understood and nor to my knowledge has a behavioural response towards these volatiles been explored using known flower visitors, apart from one study of the responses of rodents to ketones produced by flowers of a parasitic plant species (Johnson et al. 2011). It was hypothesised that an attraction towards the volatiles which arise from sugar and protein degradation would be seen for all species, as plants pollinated by rodents may emit these volatiles to enhance their attractiveness to rodents. The aim was to identify if fermentation volatiles act as behavioural cues for small mammals, and thus have the potential to assist in finding food resources.

Chapter 3: **The effects of associated fermentation and protein degradation volatiles on nectar consumption by rodents.**

Volatile compounds associated with sugar fermentation and protein degradation have been found in the volatile bouquet of plants that are pollinated by rodents (Balmer 2013). In many cases the nectar itself is the source of volatile emissions, suggesting that volatiles are present in solution in nectar. This has implications for the palatability of nectar. It was hypothesised that all rodent species would avoid consuming nectar with high volatile concentrations, either because of its unpalatability or because it would be indicative of reduced rewards due to fermentation by microorganisms. The palatability of nectar with volatiles was predicted to increase in nectar with greater sugar concentrations. The aim of this chapter was to determine how the presence of fermentation and protein degradation compounds in nectar of two differing sugar concentrations affected its consumption by rodents.

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Chapter 2: Behavioural responses of rodents to the scent of volatile signals arising from sugar and protein degradation

Abstract. Volatiles associated with sugar fermentation or protein degradation have been recorded in floral headspace samples and may thus function as a cue for pollinators. It has been reported that rodents use certain fermentation and protein degradation volatiles to find seeds and other food sources, making it plausible that these volatiles could be deployed as attractive signals by plants that rely on rodents for pollination. The present study examines the response of three rodent species (a well-known pollinator, the Namaqualand rock mouse *Micaelamys namaquensis*, an occasional floral visitor, the four- striped field mouse *Rhabdomys pumilio*, and a congener, the mesic four-striped field mouse *Rhabdomys dilectus*) to the fermentation volatile compounds ethanol, ethyl acetate and acetic acid, and the protein degradation volatile dimethyl disulphide. A traditional Y-maze choice apparatus was used to analyse the behavioural responses of rodents to these volatiles diluted at varying concentrations (0.003 – 3.0 %) in water. Rodent species differed in their responses to the four volatiles tested. Ethanol emerged as an attractant for all of the species, with the strongest response seen at the 0.3 % concentration, while only *R. dilectus* responded positively to dimethyl disulphide. Acetic acid and ethyl acetate were generally not attractive to rodents. I conclude that ethanol released through sugar fermentation has the potential to act as a behavioural cue for rodent foraging and that further studies which reveal the effect of nectar degradation by microorganisms are needed to help in understanding plant-pollinator interactions.

Plants employ a variety of sensory signals that manipulate the behaviour of potential pollinators in order to maximize the pollination function of flowers (Faegri and van der Pijl 1979; Johnson 2006; Wright and Schiestl 2009). These signals usually elicit food-seeking behaviour by animals, although other behaviours, associated with seeking sexual partners and oviposition sites, are also exploited by some plants (Schiestl 2005; Johnson 2006). Visual signals, including size, colour and shape, reflect the visual systems of pollinators (Manning 1956; Dafni and Kevan 1997). Additionally plants exploit the olfactory preferences of pollinators by emitting volatiles that attract pollinators from a distance (Faegri and van der Pijl 1979; Wright and Schiestl 2009). Volatiles can be combined with visual cues to enhance pollinator attraction and ensure that pollinators efficiently disperse pollen among plants both within and between populations (Pichersky and Gershenzon 2002; Raguso 2008).

The significance of plant volatiles is perhaps best seen in night-active pollination systems when visual cues become less reliable (Jürgens et al. 2002; Raguso and Willis 2002). For example, plants pollinated by moths, such as *Silene* species (Caryophyllaceae), produce floral volatiles, including linalool and benzyl alcohol, that are released during nocturnal floral anthesis when moth pollinators are most active (Ellis and Ellis-Adam 1993; Jürgens et al. 2002; Young 2002). Similarly flowers pollinated by rodents release pungent yeasty odours during nocturnal floral anthesis, exploiting the nocturnal activities of rodents (Wiens and Rourke 1978; Wiens et al. 1983).

Past research has identified a wide variety of volatiles released by plants that exploit innate preferences of pollinators (Raguso 2001, 2008; Wright and Schiestl 2009; Schiestl 2010). In particular, these volatiles exploit pre-existing biases, such as the responses of males to female sex pheromones that are exploited by sexually deceptive orchids (Schiestl 2005). Steenhuisen et al. (2012a) justified scent as a reason for pollination shifts in the proteas of South Africa. Specific reference was made to rodent pollination and the presence of dimethyl disulphide, a known component in the urine of mice (Singer et al. 1976; Lin et al. 2005), in headspace analysis of protea flowers visited by rodents (Steenhuisen et al. 2012a).

Scent recognition in pollinators is an effective cue during plant-pollinator interactions as the scent of a plant can be attractive to a pollinator and provide information about a plant's condition. For example, volatile compounds could warn potential pollinators about reward degradation, as in the case of infection of nectar by microorganisms (Herrera et al. 2008; Vannette et al. 2012). Unicellular organisms, such as yeasts and bacteria, metabolise sugars and possibly proteins in nectar and in doing so they release volatiles (Herrera et al. 2008; Vannette et al. 2012). Volatiles associated with sugar and protein degradation include volatile acids, esters, alcohols and sulphur-containing compounds (Wood 1961; Janzen 1977; Madigan and Martinko 2006).

Historical and current investigations have provided insight into how certain volatiles could be influencing animal foraging behaviours (Janzen 1977; Herrera et al. 2008; Vannette et al. 2012; Paulsen et al. 2013), as microorganisms colonise the food of a variety of animals; insects (Omura and Honda 2003), birds (Eriksson and Nummi 1982; Mazeh et al. 2008) and bats (Sánchez et al. 2004, 2006). Recent studies have found certain fermentation compounds to be present in the aromatic

bouquets of plants visited by rodents, potentially influencing plant-pollinator interactions (Wells et al. 2011; Steenhuisen et al. 2012a; Balmer 2013). Plants pollinated by rodents are described as having "pungent" and "yeasty" aromas, released during nocturnal floral anthesis, hinting at the potential for fermentation volatiles to be used as behaviour cues by small mammal pollinators for locating nectar or pollen rewards (Wiens and Rourke 1978; Wiens et al. 1983; van Tets 1997; Johnson et al. 2001). The attraction of rodents solely to volatiles associated with fermentation is not well understood and little of the behavioural response towards these volatiles is known. Could the fermentation volatiles arising from sugar and protein degradation be used as behavioural cues by small non-flying mammals?

The present study examines the behavioural response of three rodent species (*Micaelamys namaquensis*, *Rhabomys pumilio* and *R. dilectus*) to varying concentrations of four fermentation volatiles - ethanol, ethyl acetate, acetic acid and the protein degradation volatile dimethyl disulphide. It was hypothesised that an attraction to volatiles which arise from sugar and protein degradation would be evident for all species, as plants pollinated by rodents may emit these volatiles to enhance their attractiveness to rodents. I also hypothesized that any attraction of rodents to fermentation or protein degradation volatiles would be reduced at high volatile concentrations as greater concentrations of these volatiles in nature would be associated with reduced rewards due to the conversion of sugars or proteins by microorganisms. The aim was to identify if fermentation volatiles act as behavioural cues for small mammals, and have the potential to assist in finding food resources.

Materials and methods

Study species

The present study examines the response of three rodent taxon. First, the Namaqualand rock mouse *Micaelamys namaquensis* is a known rodent pollinator (Wiens and Rourke 1978; Wiens et al. 1983; van Tets 1997; Wester et al. 2009; Hobbhahn and Johnson 2013). It is distributed throughout southern Africa except for the Namib Desert (Nowak 1999) and is commonly found in open woodland and rocky outcrops (Skinner and Chimimba 2005). Second, the "Cape" four-striped field mouse *Rhabdomys pumilio* is an occasional floral visitor (Rourke and Wiens 1977). This species is geographically limited to the western part of South Africa (Roberts 1951; Nowak 1999; Skinner and Chimimba 2005). A second taxon of *Rhabdomys* was also used in experiments: *R. dilectus* is similar to *R. pumilio* but is considered a separate species on the basis of molecular evidence (Rambau et al. 2003). It is a seldom documented floral visitor (Johnson et al. 2011), and inhabits the moist eastern grasslands of South Africa, although its distribution tends to be patchy and discontinuous (Nowak 1999; Skinner and Chimimba 2005; Schradin and Pillay 2005).

Rodent capture and maintenance

Nine *R. dilectus* (male, n = 5; female, n = 4) were caught using baited Sherman traps at Ukulinga (27^0 67' 12" S, 30^0 40' 27" E; elevation, 680 m), the University of KwaZulu-Natal experimental farm. Forty traps were laid out and left open for 24 h for three nights in March 2013. Captured mammals were placed into individual carrier crates lined with hay and paper tissue for transportation.

Thirteen *M. namaquensis* (male, n = 6; female, n = 7) and seven *R. pumilio* (male, n = 4; female, n = 3) were caught using baited Sherman traps at Houw Hoek pass (34^0 12' 49" S, 19^0 10' 02" E; elevation, 340 m) and Sir Lowry's pass (34^0 08' 59" S, 18^0 55' 42" E; elevation, 480 m), Western Cape Province. Forty traps were laid out in each of the two locations. Traps were left open for 24 h for five nights in

July 2014. Captured mammals were placed in individual carrier crates lined with hay and paper tissue and fed full maintenance diets during transportation over two nights in August 2014 (Cape Nature permit number: 0011-AAA007-00670; KZN Wildlife permit number: OP 5166/2014). Water was provided via an overhead water feeder each night along with maintenance diet: water feeders were removed during transportation.

All animals were housed in an animal house facility, in a constant environment room with the temperature maintained at 25 ºC ± 1 ºC and a photoperiod of 14 L: 10 D. Rodents were individually housed in 30 × 30 × 50 cm glass aquaria with galvanized steel grid lids. Maintenance diets consisted of a combination seed mix, commercially available rodent pellets (AVI products, Kloof, South Africa), grated seasonal fruits and vegetables, and a combination of larval and adult meal worms. Diets were provided via a stainless steel bowl placed into the cage each evening and water was provided *ad libitum* via a stainless steel bowl, cleaned and replaced daily. Liquid activated wood pellets were provided as bedding and nesting material (AVI products, Kloof, South Africa). Shredded paper, egg cartons and cardboard rolls were offered to rodents bimonthly for enrichment. Experiments began a minimum of two weeks after capture, allowing rodents to acclimatise to the captive environment.

Procedure

To establish if volatiles associated with sugar and protein degradation elicit a behavioural response in small mammals, a traditional Y-maze choice apparatus was used (Fig. 1). Experimental volatiles consisted of varying concentrations of four volatile compounds in distilled water: ethanol (EtOH), ethyl acetate (EtOAc), and acetic acid (AcAc) each at 0.03, 0.3, and 3.0 % concentrations and dimethyl disulphide (DMDS) at 0.003, 0.03, and 0.3 % concentrations.

During the experimental trial, a mouse was placed in a glass tank (7 x 7 x 30 cm), which was attached to the Y-maze choice apparatus (Fig. 1). The Y-maze consisted of a glass tube (20 cm length, 6 cm diameter) that split into two separate arms (50 ° between each arm) (Fig. 1). The arm compartments were large enough to

allow the rodent to enter, where a fine polyester gauze separated the rodent from an experimental vial containing a control (distilled water) or an experimental volatile. A fan attached at the end of each arm blew air through the arms of the Y-maze choice apparatus (Fig. 1).

All experimental volatiles including controls were mixed, pipetted into a microcentrifuge tube (200 µl), sealed, and frozen for use on a trial day, then thawed. During trials a control was placed randomly into an arm of the Y-maze choice apparatus, with the vial containing an experimental volatile in the remaining arm, and dispersed through the apparatus. Behaviour was then recorded only if the mouse entered an arm of the Y-maze within 5 min after the scent was introduced. A positive response was recorded if the mouse's behaviour resulted in it passing an a priori marker point on an arm of the Y-maze and spending a minimum of 5 seconds investigating the scent. A negative response was defined as the same behaviour for the control arm of the Y-maze choice apparatus. A mouse was exposed to each experimental volatile four times, in order to reduce the effect of side bias (Jackson et al. 1998), with animals and experimental volatiles used in random order (i.e. 29 animals x 12 scents x 4 replicates).
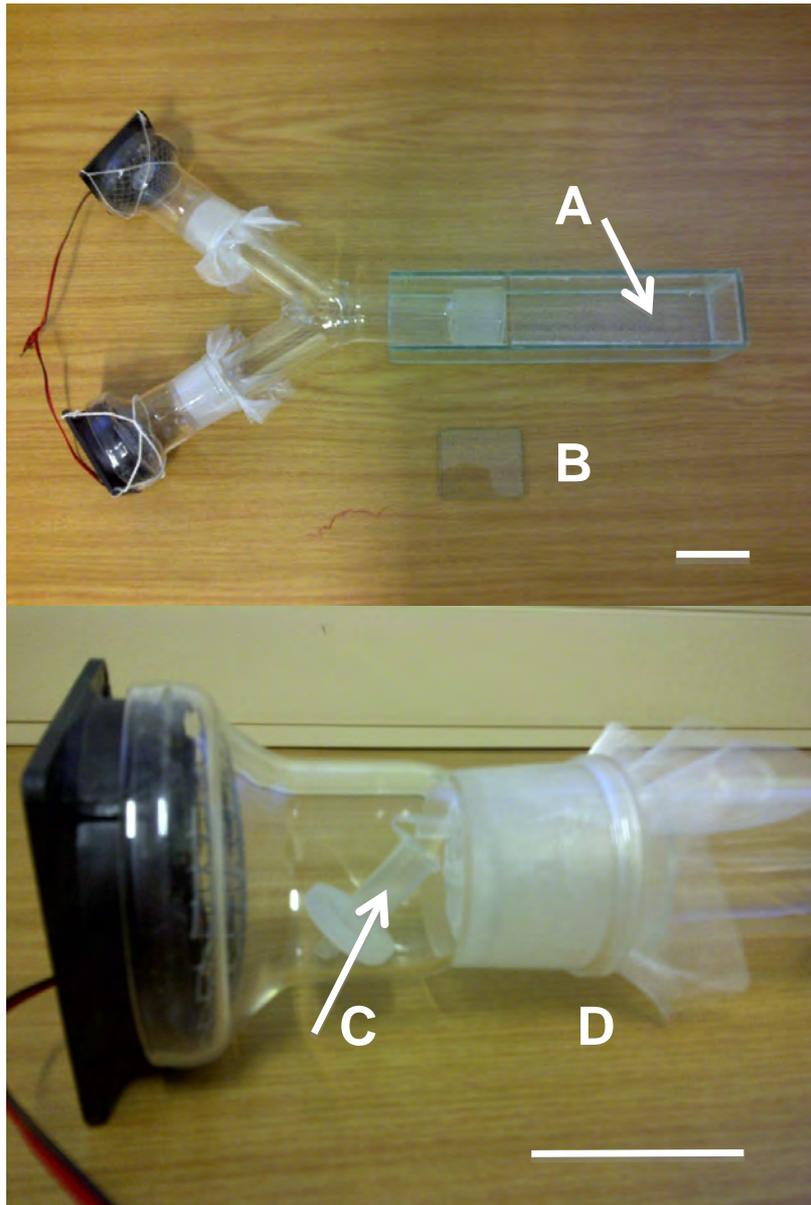
Figure 1: Y-maze apparatus set up with a volatile compound presented to a rodent in one of the arms, with a control (distilled water) in the other arm. (A) Rodents were introduced to the apparatus through the use of a holding chamber; (B) A glass sliding door was removed as the trial began, to allow the rodent access to the apparatus, Bar = 50 mm; (C) A microcentrifuge tube contained either a control or experimental volatile compound in one of the Y-maze arms; (D) Fine polyester gauze was used to stop rodents from coming into contact with the experimental volatiles, Bar = 50 mm.

Statistics

The response of rodents to volatile compounds was compared using generalized estimating equations (GEEs) to account for repeated measures of the same rodent and to accommodate non-Gaussian data in a generalized linear modelling framework. Means represented the proportion of the responses (animals that moved into the y maze) in favour of the scented arm of the Y-maze. Models for these proportions were based on a binomial distribution. Volatile concentration was treated as a covariate with volatile, species and sex as fixed effects. Interactions between these predictors were included to test whether volatile preference by a rodent species is dependent on the type of volatile or volatile concentration. Models incorporated an exchangeable correlation matrix and significance of effects was tested using Wald statistics.

Post-hoc comparisons were conducted using the sequential Sidak method. I used 95 % confidence intervals to assess whether the mean positive response towards fermentation volatiles differed significantly from 50:50 ratio (equal choice). The slope of rodent response to volatile concentration as a covariate assisted in identifying significant reduction in preference at varying concentrations. All statistical analyses were performed in SPSS 22 (IBM Corp.).

Results

M. namaquensis, *R. pumilio* and *R. dilectus* differed significantly in their responses to the volatile compounds presented to them during Y-maze choice experiments (Species, χ^2 = 28.55, df = 2, p < 0.001). The overall response of rodents towards a volatile was not affected by its concentration (Concentration, χ^2 = 1.49, df = 1, p = 0.222), or the sex of the rodents (Sex, χ^2 = 0.203, df = 1, p = 0.653), but differed significantly according to the type of volatile offered to the rodent (Volatile, χ^2 = 40.11, df = 3, p < 0.001; Fig. 2), the interaction between the type of volatile and its concentration (Volatile × Concentration, χ^2 = 18.16, df = 4, p < 0.001; Fig. 3) and the interaction between the species, the type of volatile and its concentration (Species × Volatile × Concentration, χ^2 = 13.55, df = 6, p < 0.05; Table 1).

All species were found to respond uniquely to the experimental volatiles presented to them (Species × volatile, χ^2 = 150.42, df = 11, p < 0.001; Fig. 3). Ethanol emerged as an attractant for all of the species (Fig. 2), with the strongest response seen at the 0.3 % ethanol concentration (EtOH, β = - 0.118, χ^2 = 2.317, df = 1, p = 0.128; Fig. 3a). *Rhabdomys dilectus* showed the greatest preference for ethanol, selecting higher ethanol concentrations (β = +0.210, χ^2 = 6.092, df = 1, p < 0.05), followed by *M. namaquensis* and *R. pumilio* respectively. Unlike ethanol, ethyl acetate was not preferred by all species, at all concentrations (Figs 2 and 3c). Although an overall increase in preference for higher concentrations was found (EtOAc, β = + 0.123, χ^2 = 6.471, df = 1, p < 0.05), *M. namaquensis* was significantly deterred when presented with the higher concentrations of ethyl acetate (β = - 0.171, χ^2 = 4.110, df = 1, p < 0.05; Figs 2 and 3c).

An overall drop in attractiveness of dimethyl disulphide at higher concentrations was found (DMDS, β = - 0.033, χ^2 = 0.323, df = 1, p = 0.570; Fig. 3d). Only one of the rodent species – *R. dilectus* – responded positively towards dimethyl disulphide at the lowest 0.003 % concentration (Fig. 3d), while *M. namaquensis* avoided the lowest 0.003 % and highest 0.3 % concentrations of dimethyl disulphide. Acetic acid at higher concentrations was generally found to be preferred by rodents (AcAc, β = + 0.251, χ^2 = 8.881, df = 1, p < 0.005; Fig.3b), however the apparent avoidance of *M. namaquensis* at higher concentrations of acetic acid is an interesting result (β = - 0.447, χ^2 = 15.225, df = 1, p < 0.001; Fig. 3b).
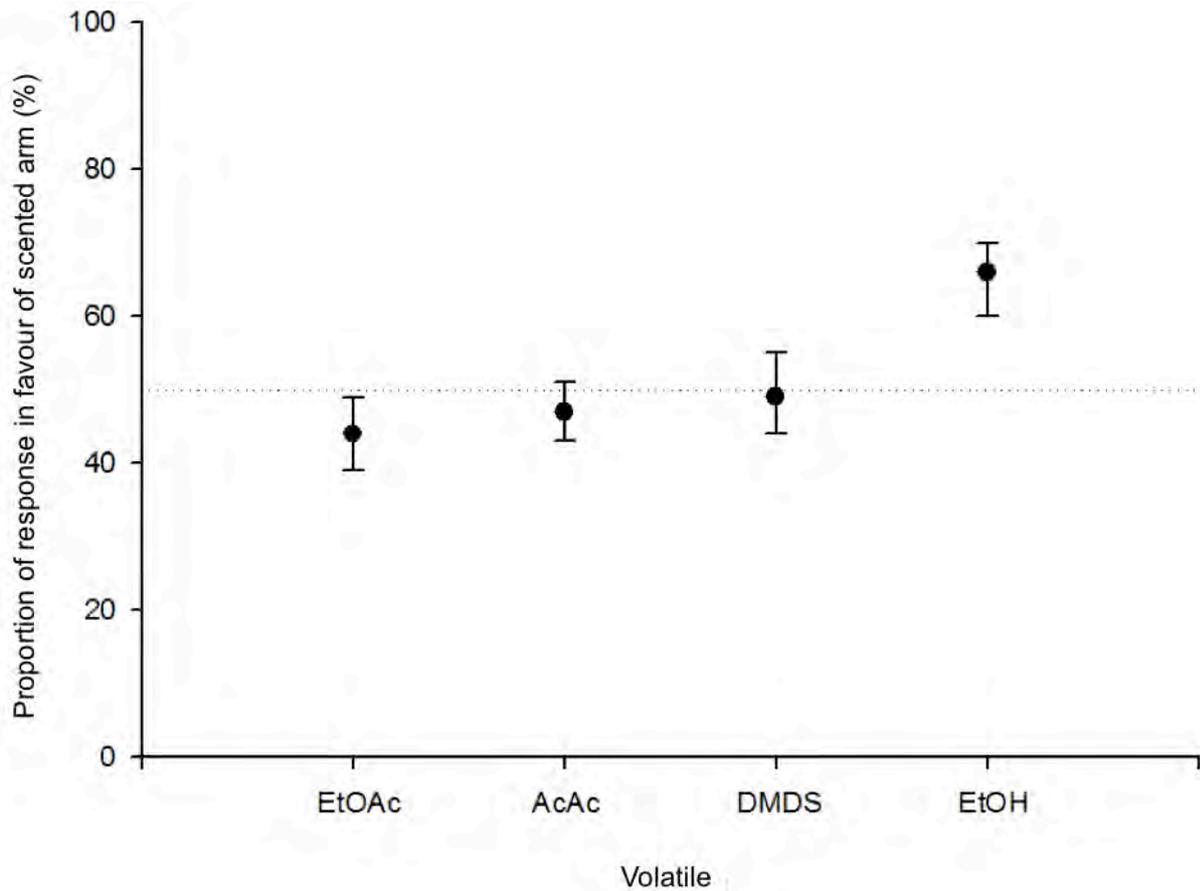
Figure 2: Marginal means (± 95 % CI) data for volatile sensitivity of all species during Y-maze experiments. Data represent the number of times all of the rodents passed an a priori marker point on an arm of the Y-maze containing a volatile compound at all of its concentrations; Ethyl acetate (EtOAc), Acetic acid (AcAc), Dimethyl disulphide (DMDS) or Ethanol (EtOH). Confidence intervals that lie above the 50% line indicate overall selection for volatile by rodents.
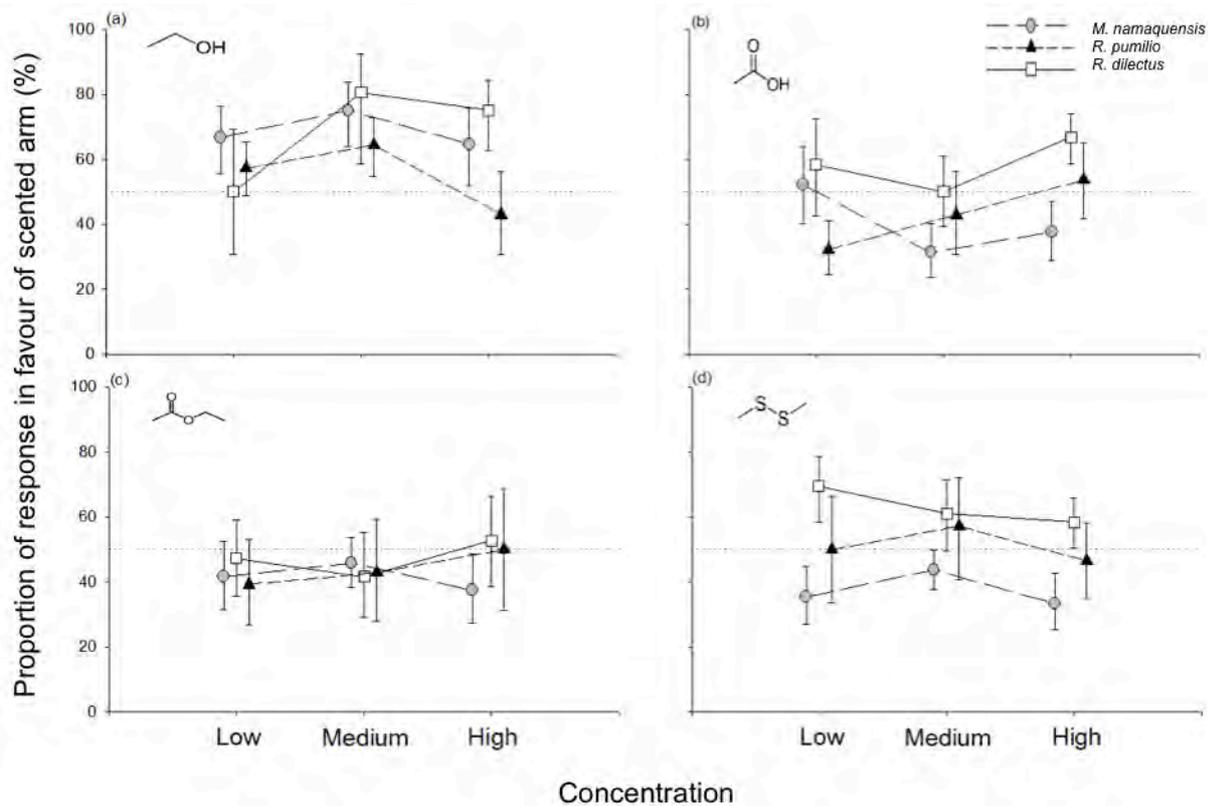
Figure 3: Marginal means (± 95 % CI) data for volatile sensitivity of *M. namaquensis* (n=13, grey circles), *R. pumilio* (n=7, black triangles) and *R. dilectus* (n=9, white squares) during Y-maze experiments. Data represent the number of times a rodent passed an a priori marker point on an arm of the Y-maze containing an experimental volatile. Rodents were offered a choice of a Low (0.03 %), Medium (0.3 %) or a High (3.0 %) concentration for (a) Ethanol (β = - 0.118, χ^2 = 2.317, df = 1, p = 0.128), (b) Acetic acid (β = + 0.251, χ^2 = 8.881, df = 1, p < 0.005) and (c) Ethyl acetate (β = + 0.123, χ^2 = 6.471, df = 1, p < 0.05). Three additional concentrations of Low (0.003 %), Medium (0.03 %) or High (0.3 %) were tested for (d) Dimethyl disulphide (β = - 0.033, χ^2 = 0.323, df = 1, p = 0.570). Horizontal line shows 50 %, indicating no selection for a volatile compound.

Table 1: Summary of the full statistical model used, indicating interaction between species and volatile, species and volatile concentration, volatile and volatile concentration, and species, volatile and volatile concentration for Y-maze experiments. Volatile concentration is always analyzed as a covariate. Bold indicates a significant difference.

Source	Wald χ²	df	Sig.
Species	11.92	2	**<0.05**
Volatile	36.13	3	**<0.01**
Concentration Covariate	1.56	1	0.212
Species × Volatile	25.98	6	**<0.001**
Species × Concentration	2.57	2	0.277
Volatile × Concentration	15.29	3	**<0.005**
Species × Volatile × Concentration	13.55	6	**<0.05**

Discussion

This study shows that rodents respond positively to a subset of the volatiles arising from sugar and protein degradation (Fig. 2). Ethanol at varying concentrations emerged as an attractant for all species, with the most significant attraction seen at the 0.3 % concentration, while only *R. dilectus* responded positively to dimethyl disulphide, with attraction seen at the 0.003 % concentration (Fig. 3).

The prediction that lower concentrations of volatile would be favoured by rodents is not supported; instead rodent responses to volatile concentrations depended on the type of volatile. Volatiles released by the food of rodents are well documented in seed detection, where rodents use the volatile emissions from seeds as scent cues to aid in seed location (Hollander et al. 2012; Paulsen et al. 2013). Desert hamsters (*Phodopus roborovskii*) were found to use seed volatiles – assumed to be ethanol, acetic acid and sulphur compounds – to effectively locate buried seeds under laboratory conditions (Paulsen et al. 2013). The attraction for rodents of ethanol, acetic acid and dimethyl disulphide at varying concentrations supports the possibility that rodents could be using volatile compounds arising from nectar degradation as a potential indicator of food resources. This is possible as the presence of these volatiles in nectar is indicative of rewards, although reduced (Herrera et al. 2008).

Recent insights into third-party interactions have described the microorganisms in nectars as being disruptive to plant-pollinator mutualisms (Herrera et al. 2008; Vannette et al. 2012). The presence of fermentation volatiles in a floral bouquet is associated with the metabolic processes of microorganisms converting carbohydrates into gases and alcohols under anaerobic conditions (Reece et al. 2011). Therefore the emission of a volatile indicative of fermentation may indicate a potential reward for the rodents, but could also indicate that much of the reward has been consumed by microorganisms. However, although floral resources may be reduced, the flowering of these plants during the winter season when other food resources are scarce may be enough to instigate the investigation of the volatiles by rodents (Rebelo 2001; Manning 2009).

Over all rodents did not select for dimethyl disulphide (with the exception of *R. dilectus* which was attracted to dimethyl disulphide at the lowest 0.003 % concentration). This was unexpected, as dimethyl disulphide is emitted by flowers of rodent-pollinated plants such as *Protea amplexicaulis* and *P. humiflora* (Steenhuisen

et al. 2012b; Balmer 2013). Earlier studies looking at the social behaviour of mice found that dimethyl disulphide is a component in the urine of male mice, as well as being a component in female vaginal secretions of golden hamsters *Mesocricetus auratus* (Singer et al. 1976; Lin et al. 2005). Both studies suggest the potential for dimethyl disulphide to play a role in mammalian social behaviour. A later revaluation of the bioassays employed by Petrulis and Johnston (1995) suggested that earlier findings were due to an inaccurate biological bioassay and concluded that *M. auratus* was not attracted to dimethyl disulphide alone (Johnston 2003). Current findings suggest that dimethyl disulphide attraction may also be concentration dependant, but more importantly is dependent on the presence of other compounds enhancing overall attraction (Johnston 2003; Steenhuisen et al. 2012a; Balmer 2013).

Alcohol and sulphur containing compounds have been found in the volatile components of *Nepenthes rajah*, whose scent was described as resembling the odour of cabbage (Wells et al. 2011). These pitchers are cryptic in nature, often hidden within dense grassy vegetation, and are visited by small mammals who defecate into the pitchers as they feed from extrafloral nectar on the pitcher lids (Greenwood et al. 2011; Wells et al. 2011). The cryptic and pungent smelling structures suggest similarities between rodent visitation of the Bornean pitcher plants and rodent pollination in South Africa (Wiens et al. 1983; Johnson et al. 2001; Greenwood et al. 2011; Wells et al. 2011). However the interaction between mammals and *N. rajah* is described as being one of resource exchange on a mutualistic basis – plant nectar for small mammal droppings – providing the plant with much of its nitrogen and phosphorus requirements, not for pollination (Greenwood et al. 2011). Although the similarities in scent between rodent pollinated proteas and *N. rajah* hint at the potential that small mammals could be using the volatiles arising from protein degradation as an indicator of food resources (Greenwood et al. 2011; Wells et al. 2011; Steenhuisen et al. 2012a), the role of dimethyl disulphide in the floral bouquet of rodent-pollinated plants remains something of a mystery.

The results also showed that rodents were significantly repelled by ethyl acetate and that it was the least preferred compound for all species (Figs 2 and 3c). This result is potentially linked to the formation of ethyl acetate, which is produced at a later stage of fermentation. Ethyl acetate is an ester of ethyl alcohol and acetic acid

and can only form once both are present (Swiegers et al. 2005; Reece et al. 2011). Therefore ethyl acetate, a volatile present in beetle pollinated proteas, may be indicative of a later stage of fermentation and may suggest to rodent visitors that a further reduction in rewards will be found (Bernhardt 2000; Steenhuisen and Johnson 2012; Steenhuisen et al. 2012c).

Whilst conducting the Y-maze experiments I was interested to note the frequent similarity in the behavioural responses of *M. namaquensis* and *R. pumilio* to fermentation compounds. This is of interest as previous studies have found that *M. namaquensis* is a frequent floral visitor and pollinator of certain *Protea* species (Wiens et al. 1983; van Tets 1997), whereas *R. pumilio* is an occasional floral visitor quite destructive in its floral visitation (Rourke and Wiens 1977). However both species have been recorded visiting rodent pollinated flowers: they may come into contact with these or similar fermentation compounds more often than current research shows (Biccard and Midgley 2009; Steenhuisen et al. 2012b; Balmer 2013).

Rhabdomys dilectus showed the strongest attraction towards all the fermentation volatiles presented to them during the Y-maze experiments. This finding may be related to the foraging habit and food resource utilization of these animals. *Rhabdomys dilectus* has been documented feeding on the nectar of flowers (Johnson et al. 2011), however is more commonly noted as being a generalist omnivore, feeding on a wide range of plants including the fruit pods of certain trees (Skinner and Chimimba 2005). The fruits that *R. dilectus* feeds on may be fermented to some degree (Janzen 1977; Knudsen et al. 2006), resulting in the trends seen during the Y-maze experiments.

The role of volatiles released by flowers and fruits which arise from sugar and protein degradation is surprisingly poorly investigated for small non-flying mammals compared to bats or invertebrates (Bestmann et al. 1997; Bernhardt 2000; Steenhuisen and Johnson 2012; Steenhuisen et al. 2012c), even though small mammals are well studied in terms of their olfactory system (Tirindelli et al. 2009; Paulsen et al. 2013). Von Helversen et al. (2000) tested how nectar-feeding bats would respond to single floral volatiles from bat pollinated plants. They found that bats were able to successfully orientate themselves during food location guided only by scent. Sulphur compounds, specifically dimethyl disulphide, proved to be especially important in bat orientation, providing insight into why over 70 % of bat

pollinated plant nectars are known to contain sulphur compounds (Knudsen and Tollsten 1995; Bestmann et al. 1997).

Considering the functional role of a volatile compound in a rodent pollinated plant, only one study has conclusively shown that a single volatile compound can be attractive to rodent pollinators. Johnson et al. (2011) looked at the volatile components of a parasitic plant (*Cytinus visseri*), and found that the ketone 3-hexanone, one of the three major volatile components that made up the scent of the flower, was attractive to *R. dilectus* in Y-maze experiments.

In summary, ethanol was found to be attractive to all rodent species and greater selecton for this volatile is seen at medium concentrations. Volatiles indicative of later stage fermentation were avoided by all species, and only *R. dilectus* showed a positive response towards dimethyl disulphide. The differences seen in rodent responses to volatile compounds between the two *Rhabdomys* species support the justification for a key biological difference between *R. dilectus* and *R. pumilio*. The importance of ethanol and dimethyl disulphide as a potential behavioural cue needs further attention as it has the potential for evolutionary understanding of rodent pollination and this could be essential for future studies. I conclude that certain compounds released through sugar fermentation or protein degradation have the potential to act as behavioural cues in rodent pollination and that further studies which reveal the effect of nectar degradation by microorganisms are needed to help in understanding plant-pollinator interactions.

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Chapter 3: The effects of associated fermentation and protein degradation volatiles on nectar consumption by rodents

Abstract. Nectar degradation by microorganisms has the potential to alter the interactions between animal and plant mutualists, as compounds produced through sugar degradation in nectar may be perceived either as a signal of reduced rewards for flower visitors or as a signal for a food source itself. Furthermore, if present in nectar, volatiles associated with sugar and protein degradation may alter its palatability. Rodents are known to respond to fermenting food sources and to visit flowers with a scent containing fermentation and protein degradation volatiles. The present study examines the effects of four of these volatiles (ethanol, ethyl acetate, acetic acid, and dimethyl disulphide) on nectar consumption by three rodent species (a well-known pollinator, the Namaqualand rock mouse *Micaelamys namaquensis*, an occasional floral visitor, the three striped field mouse *Rhabdomys pumilio*, and a congener, *Rhabdomys dilectus*). Paired choice tests were used to identify rodent responses to experimental solutions containing volatiles. The study was designed to analyse the preference of rodents for solutions containing volatiles at varying concentrations (0.003 - 3.0 %) and sucrose at two concentrations (0.73 and 1.46 M). Both *M. namaquensis* and *R. pumilio* preferred medium (0.3 %) concentrations of ethanol in high sugar concentration diets, but found this compound distasteful in lower sugar concentration diets. Acetic acid and ethyl acetate were generally not preferred by any species, in either sugar concentration. Dimethyl disulphide was preferred by all species only when present in higher sugar concentrations (1.46 M) and at low volatile concentrations (0.003 %). Results suggest that rodents alter their choice of diet in relation to the type and concentration of compounds in an experimental diet. I conclude that emission of volatiles from experimental diet is an honest signal of nectar amount may be limited by the effects of these volatiles on nectar palatability, and that these effects are dependent on sugar concentrations in nectar.

Plants benefit from offering rewards that are attractive to prospective animal visitors (Wright and Schiestl 2009). Successful attraction is accomplished through the ability of plants to communicate with their animal pollinators by using various signals (Faegri and van der Pijl 1979; Johnson et al. 2011). These cues are effective when they are interpreted by animal visitors as being associated with rewards. Cues exploit visual or olfactory senses of pollinators in an attempt to guide floral visitors to embark on behaviour that is beneficial to pollination. Morphological cues include colour and size of flowers (Manning 1956; Lunau and Maier 1995) and physiological cues include sugar concentration or the volatiles emitted by flowers (Pichersky and Gershenzon 2002; Raguso 2008; Wells et al. 2011).

Potential rewards advertised by plants entice floral visitors to return (foraging constancy). Pollinator return could be linked to the taste of a nectar (Omura and Honda 2003; Irwin et al. 2004; Irwin and Adler 2008), or to other rewards offered by a plant (van Tets 1997; Fleming and Nicolson 2002; Davies et al. 2003). Rewards utilized by floral visitors for food and as building materials include nectar, pollen, waxes and resins all of which are advertised as being on offer by flowers (van Tets 1997; Johnson et al. 1999; Davies et al. 2003; Singer and Koehler 2004; Fenster 2006; Yuan et al. 2007; Wells et al. 2011). The taste of a nectar reward offered to floral visitors could act as a pollinator cue and has the potential to be a decisive factor in floral selection by pollinators (Baker and Baker 1982; 1983; Johnson et al. 2006; Zhang et al. 2012). Components of floral nectar include the type, relative contributions and concentrations of sugar, all of which dictate how long a visitor will remain at a particular plant before moving on in search of its next floral reward (Baker and Baker 1983). However in certain cases other compounds may be used to influence floral visitation (Baker and Baker 1982). For example, amino acids in nectar could potentially contribute to pollinator attraction and or feeding in female cabbage white butterflies *Pieris rapae* (Alm 1990). In other cases, it has been shown that the function of secondary compounds is not to attract pollinators, but rather to repel unwanted flower visitors (Johnson et al. 2006; Zhang et al. 2012).

Sometimes the rewards offered in plant-pollinator interactions are degraded by a third party, as shown in the infection of floral nectars by microorganisms (Herrera et al. 2008; Vannette et al. 2012). These third party players which include yeasts and bacteria degrade floral nectars, altering sugar composition and concentration, and the compounds in nectar through the release of fermentation volatiles as metabolic waste (Wood 1961; Madigan and Martinko 2006; Herrera et al. 2008; Vannette et al. 2012). Therefore plant-pollinator mutualisms are being altered through a reduction in floral attractiveness due to diminished floral rewards for effective pollinators and through the production of volatile compounds that are potentially unattractive, thus resulting in reduced fitness for plants and pollinators (Raguso 2004; Herrera et al. 2008). Recent insight into the effect third party players may be having on plant-pollinator mutualisms has stimulated pollination biologists to identify how the compounds arising from sugar or possible protein degradation in nectar may be influencing plant-pollinator interactions (Herrera et al. 2008; Vannette et al. 2012; Records 2012).

Fermentation compounds associated with sugar and protein degradation of nectar have been found in the volatile bouquet of plants that are pollinated by rodents (Balmer 2013). These volatile bouquets have been described in studies of rodent pollination as being perceived by humans as "yeasty" or "pungent" smelling (Wiens et al. 1983; Johnson et al. 2001). Several fermentation volatiles as well as the protein degradation product dimethyl disulphide have been isolated from the scent of flower parts and nectar of rodent-pollinated plant species in South Africa (SD Johnson, unpublished data). This has led to interest in identifying if volatiles in nectar associated with sugar and protein degradation are perceived as being indicative of diminished rewards for rodent pollinators, or if they are perceived as being a cue for actual food sources. To my knowledge no fermentation volatile compounds identified in the nectar of a rodent pollinated plant are known to influence flower selection during rodent plant-pollinator interactions, although a recent study did identify a functional role for ketones (which can be indicative of fat degradation) in the scent of a rodent-pollinated parasitic plant (Johnson et al. 2011). However, no study has included a test of the responses of rodents to volatiles in the nectar that they consume. Therefore, could fermentation volatile compounds influence nectar consumption and how could this influence plant-pollinator interactions involving rodents?

The present study examines the choices, measured in terms of consumption, among experimental diets containing four fermentation volatiles of varying concentrations – ethanol, ethyl acetate, acetic acid and dimethyl disulphide – by three rodent species (*Micaelamys namaquensis*, *Rhabomys pumilio* and *R. dilectus*). It was hypothesised that all species would show a increased selection for lower volatile concentrations in their experimental solutions, avoiding higher concentrations because they are either distasteful or perceived as indicative of reduced rewards. I also hypothesise that all species will increase their preference for volatiles and higher volatile concentrations when these are paired with greater sugar concentrations.

Materials and methods

Study species

The present study involved three rodent species. The Namaqualand rock mouse *Micaelamys namaquensis* (Wiens and Rourke 1978; Wiens et al. 1983; van Tets 1997; Wester et al. 2009; Hobbhahn and Johnson 2013) is distributed throughout southern Africa except the Namib Desert (Nowak 1999) and is commonly found in open woodland and rocky outcrops (Skinner and Chimimba 2005). The "Cape" four-striped striped field mouse *Rhabdomys pumilio* (Roberts 1951; Rourke and Wiens 1977) is an occasional floral visitor and geographically limited to the western part of South Africa (Roberts 1951; Nowak 1999; Skinner and Chimimba 2005). The eastern striped field mouse *R. dilectus* is similar to *R. pumilio* but has been suggested a separate species (Rambau et al. 2003). It is a seldom documented floral visitor (Johnson et al. 2011), and inhabits the moist eastern grasslands of South Africa (Nowak 1999; Skinner and Chimimba 2005; Schradin and Pillay 2005).

Rodent capture and maintenance

Thirteen *Micaelamys namaquensis* and seven *Rhabdomys pumilio* were caught using baited Sherman traps at Houw Hoek pass (34^0 12' 49" S, 19^0 10' 02" E; elevation, 340 m) and Sir Lowry's pass (34^0 08' 59" S, 18^0 55' 42" E; elevation, 480 m), Western Cape Province. Nine *Rhabdomys dilectus* (male, n = 5; female, n = 4) were caught using baited Sherman traps at Ukulinga (27^0 67' 12" S, 30^0 40' 27" E; elevation, 680 m), the University of KwaZulu-Natal experimental farm.

Only nine *M. namaquensis* (male, n = 5; female, n = 4) were used in taste experiments due to feeder equipment limitations, and six of the original *R. pumilio* (male, n = 3; female, n = 3) were used as one animal died before experiments could begin.

All animals were housed in an animal house facility, where they remained in a constant environment room with the temperature maintained at 25 ºC ± 1 ºC and a photoperiod of 14 L: 10 D. Rodents were individually housed in 25 × 45 × 25 cm plastic tub cages with stainless steel grid lids. Maintenance diets were made up of a

combination seed mix, commercially available rodent pellets (AVI products, Kloof, South Africa), grated seasonal fruits and vegetables, and a combination of larval and adult meal worms. Diets were provided via an overhead feeder and a seed tray placed into each rodent cage and water was provided *ad libitum* from inverted water bottles. Liquid activated wood pellets were provided as bedding and nesting material (AVI products, Kloof, South Africa). Shredded paper, egg cartons and cardboard rolls were offered to rodent's bimonthly for enrichment. Experiments began a minimum of two weeks after moving rodents into plastic tub cages, allowing rodents to acclimatise to the new environment.

Experimental diets

To investigate whether the preference of *M. namaquensis*, *R. pumilio* and *R. dilectus* for artificial diet containing volatile compounds was dependent on sugar concentration, two sucrose concentrations similar to common rodent pollinated plants were used: 0.73 M (20 % w/w) and 1.46 M (40 % w/w) (Wiens et al. 1983). Experimental diets contained varying concentrations of: ethanol (EtOH), ethyl acetate (EtOAc), and acetic acid (AcAc) each at 0.03, 0.3, and 3.0 % concentration and dimethyl disulphide (DMDS) at 0.003, 0.03, 0.3 % concentration for both sugar concentrations. Preference for an experimental diet is expressed as the ratio of solution with volatiles consumed to total solution consumed (control + experimental diet): a ratio of 50 % indicates no difference in rodent preference.

Each rodent received one diet pair, an experimental diet and a control at a matched sugar concentration. The experiment was then repeated for all experimental diets at both sucrose concentrations, each rodent receiving a total of 24 paired solutions in random order. The aim was to present each rodent with a different diet on the same night, however due to rodent numbers several rodents would have received the same diet pair on any given night.

Feeders

Throughout experiments rodents had access to two feeders, constructed from 200 ml inverted rodent glass water bottles. Bottles were sealed off using neoprene tubing, and stainless steel rodent drinking sippers (AVI products, Kloof, South Africa) were inserted through the tubing to create the rodent sipper (Fig. 1). Drinking spouts extended 20 mm into cages (Fig. 2). To protect the feeders from being chewed by rodents, a 20 mm diameter steel washer was placed on the cage lid through which the spouts were inserted (Tordoff and Bachmanov 2003). The design of the feeder ensured that evaporation losses were negligible.
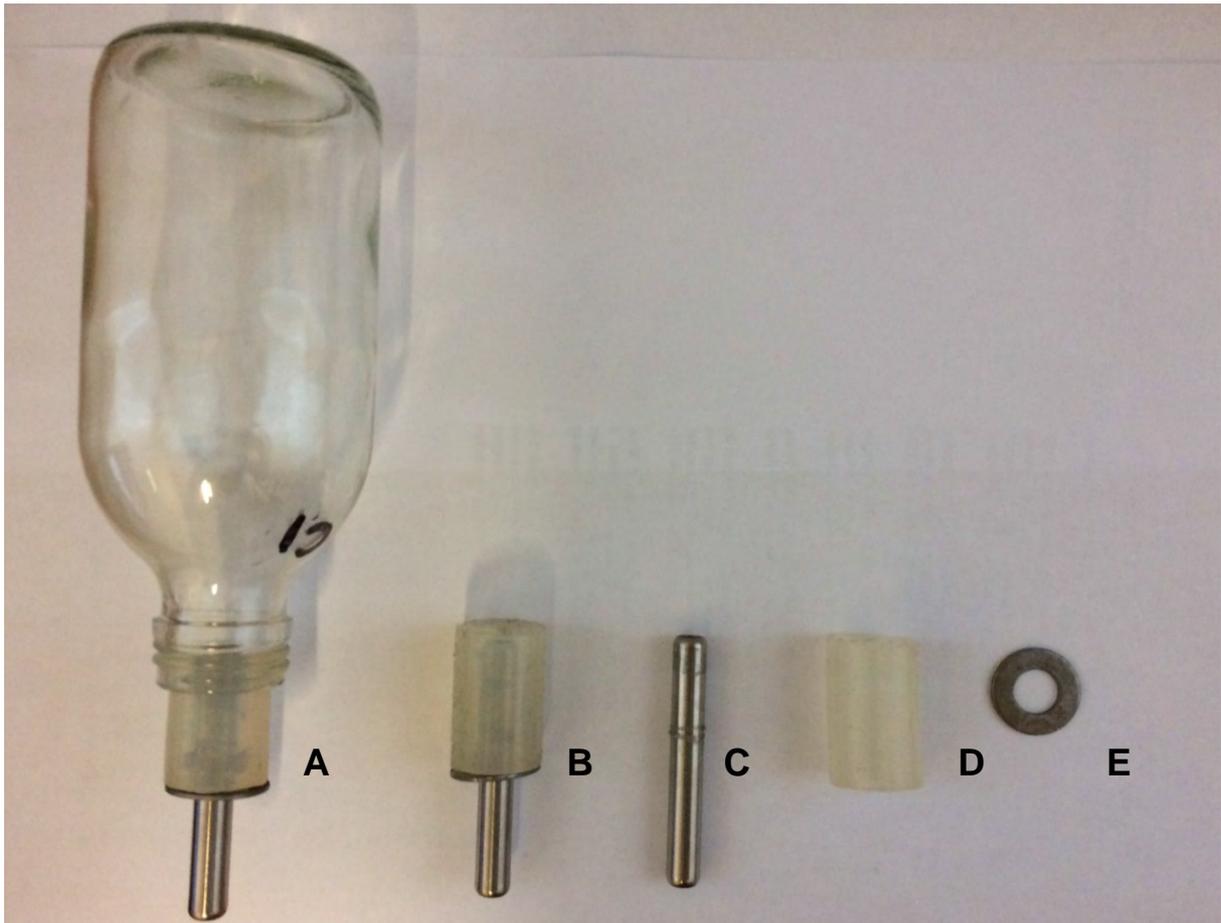
Figure 1: Rodent feeder construction. (A) a complete rodent feeder, with rodent sipper attachment; (B) The rodent sipper attachment; (C) Stainless steel rodent sipper (AVI products, Kloof, South Africa), protruded into the cage about 20 mm; (D) 20 mm neoprene stopper; (E) 20 mm diameter steel washer, to protect stopper from gnawing.

Procedure

On a trial day, experimental solutions and their paired controls were mixed, placed in rodent feeders, weighed, and placed through the stainless steel grid lids on the plastic tub cages at 15h00 (Fig. 2). Experiments started 4 h before and ended 4 h after the dark cycle, due to the crepuscular and nocturnal nature of the rodents (Skinner and Chimimba 2005). Rodent sippers of experimental diets and control solutions were separated by a 5 cm gap (Fig. 2). The trial lasted 18 h. To limit the effects of side bias, the feeder positions were reversed at 9 h: in order to reduce disturbance only one switch was performed under red light conditions (Jackson et al. 1998). After the 18 h experimental period the feeders containing experimental solutions and their paired controls were reweighed to obtain intake for each rodent. Trials were separated by at least one maintenance day; during this time rodents were fed full maintenance diets and water was provided *ad libitum* from inverted water bottles.

Figure 2: *Rhabdomys pumilio* feeding from a rodent sipper containing an experimental solution, with a paired control rodent sipper to the right. The rodent feeders were placed through the stainless steel grid lids on the plastic tub cages. Note that the experimental and control solutions are separated by a 5 cm gap.

Statistics

Preference for the solution containing volatiles was calculated as the proportion of total sugar solution consumption that was contributed by consumption of the solution with volatiles. As these values were non-binomial proportional values, the values were logit transformed and then compared using a generalized estimating equation (GEEs) to account for repeated measures of the same rodents in a generalized linear modelling framework (Warton and Hui 2011). A Gaussian distribution and identify link function was used, and rodent ID was used as a repeated measure. Volatile concentration was treated as a covariate with sugar concentration, species and sex of rodents as fixed effects. Interactions between these predictors were included to test whether consumption of volatile compounds was dependent on sugar concentration and/or volatile concentration. Models incorporated an exchangeable correlation matrix and significance of effects was tested using Wald statistics. Since the analysis of continuous proportions is known to be statistically challenging (Warton and Hui 2011) I also tested various alternative data transformations and distribution models, including models which used logit transformed data (with Gaussian distribution and identity link), arcsine transformed data (with Gaussian distribution and identity link), original data using a Gamma distribution and a log link function, or a beta distribution (with a link function using SAS) (Appendix Table 1).

Post-hoc comparisons were conducted using the sequential Sidak method. I used 95 % confidence intervals (calculated by back-transformation from the logit scale) to assess whether the mean positive response towards fermentation volatiles differed significantly from a 50:50 ratio (equal choice). Volatile concentration as a covariate was used in the comparison to assess the overall effect of volatile concentration on preference. All statistical analyses were performed in SPSS 22 (IBM Corp.).

Results

All species differed significantly in their response to the volatile compounds presented to them during taste experiments (Species, χ^2 = 7.15, df = 2, p < 0.05). Species diet selection was influenced by the type of volatile compounds (Species × Volatile, χ^2 = 371.27, df = 9, p < 0.001), volatile concentrations (Species × Volatile concentration, χ^2 = 1677.96, df = 6, p < 0.001) and the sugar concentration (Species × Sugar concentration, χ^2 = 215.50, df = 3, p < 0.001) of the experimental diets presented during taste choice experiments. *Micaelamys namaquensis* showed the greatest preference towards volatile compounds added to an experimental diet, while *R. dilectus* and *R. pumilio* were similar in their selection preferences.

The palatability of an experimental diet was not affected by the sex of the rodents (Sex, χ^2 = 0.13, df = 1, p = 0.716), but was influenced by the type of volatile (Volatile, χ^2 = 73.96, df = 3, p < 0.001), the concentration of a volatile (Volatile concentration, χ^2 =292.54, df = 2, p < 0.001) as well as the interaction between volatile and volatile concentration (Volatile × Volatile concentration, χ^2 = 46.44, df = 6, p < 0.001) of the experimental diets presented to the rodents. Rodents showed an increased consumption of experimental diets containing lower concentrations of volatiles (β = - 0.390, χ^2 = 25.82, df = 1, p < 0.001).

The sugar concentration of an experimental diet influenced rodent choice (Sugar concentration, χ^2 = 147.29, df = 1, p < 0.001), with increased selection towards volatiles when volatiles were combined with the higher 1.46 M sugar concentration (Volatile × Sugar concentration, χ^2 = 13.88, df = 3, p < 0.005). The interaction between sugar concentration and volatile concentration (Sugar concentration × Volatile concentration, χ^2 = 843.95, df = 5, p < 0.001) had a significant effect on rodent preference (β = 0.610, χ^2 = 147.29, df = 1, p < 0.001), as well as the interaction between volatile, volatile concentration and sugar concentration of an experimental diet (Volatile × Volatile concentration × Sugar concentration, χ^2 = 220.54, df = 17, p < 0.001). Increased consumption was seen for experimental diets containing low volatile concentrations paired with high sugar concentrations.

As the volatile concentration in rodent experimental diets increased, preference for an experimental diet containing volatile compounds decreased at both sugar concentrations (β = - 0.111; χ^2 = 51.14, df = 9, p <0.001; Fig. 3; Table 1), with an exception seen for ethanol at the medium 0.3 % concentration for *M. namaquensis* and *R. pumilio* (Fig. 3a).

Ethanol and dimethyl disulphide were avoided by all species at both sucrose concentrations, which was less pronounced for the lower volatile concentrations; however a significant increase in preference was seen at the higher 1.46 M sugar concentration (χ^2 = 13.88, df = 3, p = 0.003). At the higher sucrose concentration diet *M. namaquensis* and *R. pumilio* showed a preference for ethanol for the intermediate concentration and for a low concentration of dimethyl disulfide (Fig. 3a). All species showed a preference for the low 0.003 % dimethyl disulphide solution added to the greater 1.46 M sugar experimental diet (β = - 0.697, χ^2 = 130.22, df = 1, p < 0.001; Fig. 3d). This volatile was repellent in greater concentrations for both sugar concentrations (Fig. 3d).

Ethyl acetate and acetic acid were not preferred by any species at both sucrose concentrations, however a significant increase in preference was seen at the lower volatile concentrations for acetic acid (AcAc, β = - 0.341, χ^2 = 30.68, df = 1, p < 0.001) and ethyl acetate (β = - 0.258, χ^2 = 11.36, df = 1, p = 0.001; Fig. 3c). All concentrations of ethyl acetate were significantly rejected by all rodent species at the low 0.73 M sugar concentration experimental diets (Fig. 3c).

Additional data transformations and distribution models showed that the logit, arcsine and gamma distribution models gave very similar results, but the beta distribution showed some contrasting results. In particular, the overall model for the beta distribution differed from the others in being not being significant (χ^2 = 504, df = 18, p = 0.0753), but most of the individual effects were congruent with the other models (Appendix table 1).
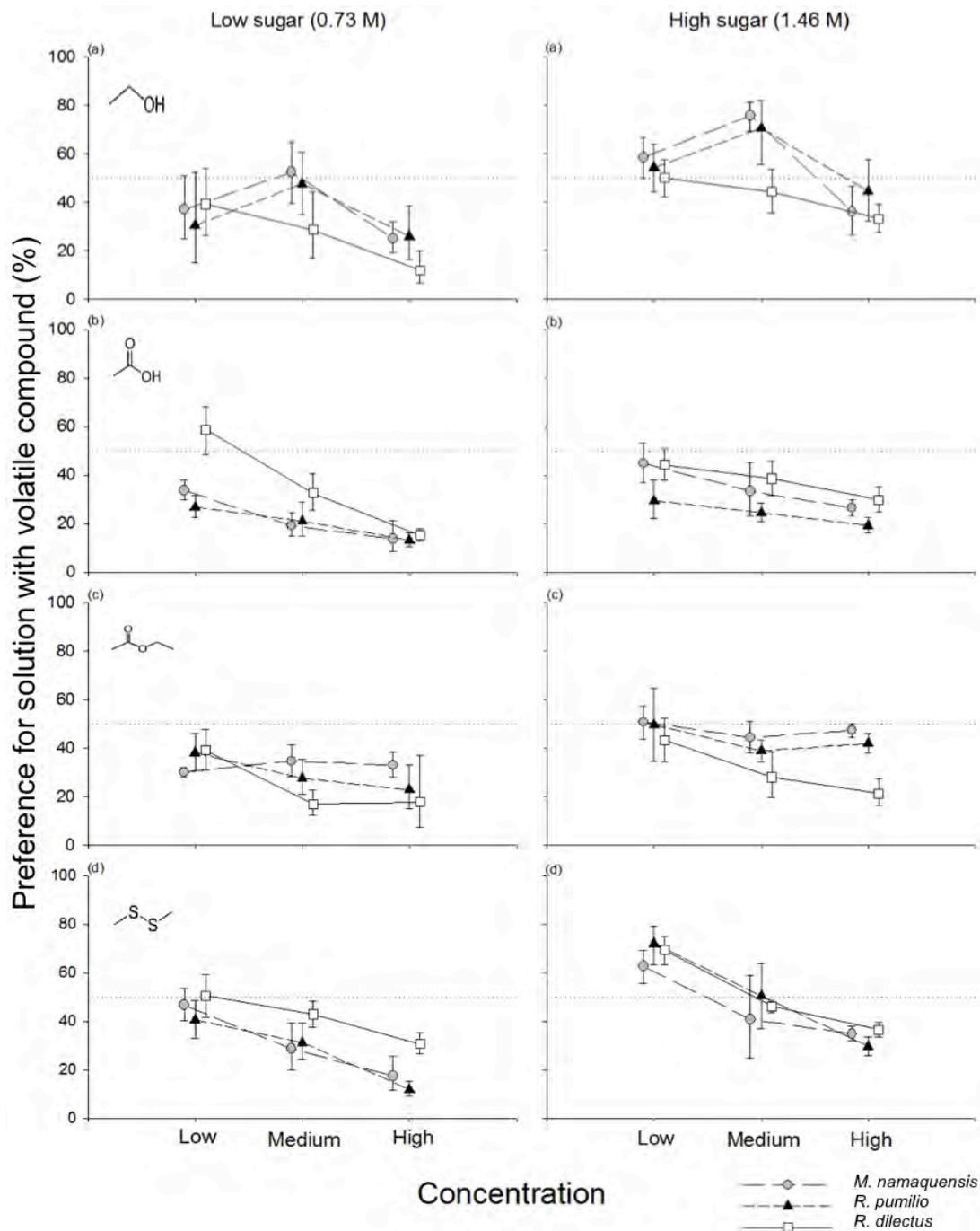
Figure 3: The mean (± 95 % CI) preference of rodents for sugar solutions containing a volatile compound for *M. namaquensis* (n=9, grey circles), *R. pumilio* (n=6, black triangles) and *R. dilectus* (n=9, white squares) during taste experiments. Rodents were offered a choice of a low (0.73 M) or a high (1.46 M) sucrose solution with or without a Low (0.03 %), Medium (0.3 %) or a High (3.0 %) concentration for (a) Ethanol, (b) Acetic acid and (c) Ethyl acetate. Three additional concentrations of Low (0.003 %), Medium (0.03 %) or a High (0.3 %) were tested for (d) Dimethyl disulphide. A preference value of 50 %, indicated by the dotted line, indicates no discrimination between the sugar solutions containing a volatile compound and a control sugar solution.

Table 1: Summary of the full statistical model used of logit transformed data, indicating all interactions for taste choice test experiments. Bold indicates a significant difference.

Source	Wald χ^2	df	Significance
Species	7.145	2	**<0.05**
Volatile	123.844	3	**<0.001**
Volatile Concentration	631.478	2	**<0.001**
Sugar Concentration	201.192	1	**<0.001**
Species × Volatile	142.386	6	**<0.001**
Species × Volatile Concentration	44.821	4	**<0.001**
Species × Sugar Concentration	11.508	2	**<0.001**
Volatile × Volatile Concentration	95.237	6	**<0.001**
Volatile × Sugar Concentration	28.869	3	**<0.001**
Sugar Concentration × Volatile Concentration	2.703	2	0.259
Species × Volatile × Volatile Concentration	123.598	12	**<0.001**
Species × Sugar Concentration × Volatile	35.857	6	**<0.001**
Species × Sugar Concentration × Volatile Concentration	5.928	4	0.205
Species × Sugar Concentration × Volatile × Volatile Concentration	139.728	17	**<0.001**

Discussion

The functional roles of volatile compounds in the nectar of rodent pollinated plants remain little investigated. This study shows how a subset of the volatiles associated with the degradation of nectar by microorganisms could influence nectar consumption by rodent floral visitors. The diet choice of rodents were dependant on the type and concentration of volatile compounds added to experimental diets of varying sugar concentrations (Fig. 5). Despite not functioning as a long distance attractant for *M. namaquensis* and *R. pumilio* (Chapter 2), dimethyl disulphide at varying concentrations was preferred by all species when added to the higher concentration (1.46 M) experimental diet. Both *M. namaquensis* and *R. pumilio* responded positively to ethanol, with the greatest preference seen at the 0.3 % concentration added to the greater sucrose concentration (Fig. 5).

The occurrence of microorganisms in floral nectar is a consequence of floral visitors transferring various unicellular organisms between nectars (de Vega and Herrera 2012). As a result the sugar concentration and composition of floral nectars are modified by these microorganisms and this could potentially influence pollinator behaviour (Goodrich et al. 2006; Madigan and Martinko 2006; Herrera et al. 2008; 2009). The presence of volatiles associated with nectar and protein degradation in nectar alters the taste of experimental diets, therefore having the potential to act as a pollinator cue, and may be a decisive factor in floral selection during pollination (Johnson et al. 2006; Zhang et al. 2012). This is in accordance with previous studies which have shown that the function of certain secondary compounds in nectar is not to attract pollinators, but rather to repel unwanted flower visitors (Johnson et al. 2006; Zhang et al. 2012). The taste experiments showed that rodents avoided lower volatile concentrations less than volatiles greater in concentrations. This suggests that rodents may perceive that floral visitation has already taken place and rewards may be reduced due to microorganism activity (Madigan and Martinko 2006; Herrera et al. 2008; Vannette et al. 2012). On the other hand, it may be a simple consequence of rodents finding the volatiles distasteful.

Sugar concentrations in the nectar of rodent pollinated plants are greater than those found in bird pollination systems (Wiens et al. 1983; Johnson and Nicolson 2008), and a reduction in sugar concentration due to microorganism infection has the potential to significantly alter rodent floral visitation. Rodent pollinated Proteaceae

have high sucrose in their nectars (Nicolson and van Wyk 1998) so there is more potential for degradation from sucrose to hexose, and rodents may not enjoy hexose sugars in their nectar (Maller and Kare 1965; Feigin et al. 1987; Johnson et al. 1999). The taste experiments showed that sugar concentration had a significant effect on rodent responses to volatile compounds, with the preference for volatiles increasing at greater sugar concentrations of the experimental diets. This could be an indication that mice use the taste of their nectar as a cue to the state of microbial infection. More importantly greater sugar concentrations were able to increase rodent preference for a volatile: this can be compared with the response of nectar-feeding birds to nicotine in different sucrose concentrations (Lerch-Henning and Nicolson 2013). Consequently the taste of a volatile compound in dilute nectars enables rodents to reject flowers due to reward degradation by microorganisms. De Vega et al. (2009) showed that there is a negative correlation between yeast density and sugar concentration in floral nectars. Additionally, Herrera et al. (2008) found that yeast cell density increased as flowers progressed through floral stages and that the presence of yeasts caused intraspecific patchiness of nectar characteristics, with the potential to alter pollinator behaviour. greater sugar concentrations in the presence of volatile compounds should therefore increase the preference for degraded nectars as high sugar concentration is indicative of lower microorganism densities. Therefore rodent preference for lower concentrations of volatile compounds paired with greater sugar concentrations in experimental nectars could be linked to their perception of floral nectar quality.

The type of volatile compound had a significant effect on diet preference, with rodents showing an increased preference for diets containing ethanol or dimethyl disulphide over those with acetic acid and ethyl acetate. Balmer (2013) considered the scent of three nonflying mammal pollinated proteas and found that alcohols and dimethyl disulphide made up the majority of their floral bouquets. Her results suggest that rodents may be adapted to ethanol and dimethyl disulphide at varying concentrations in their nectars (Steenhuisen et al. 2012; Balmer 2013), as has been suggested for other elements of the diet of rodents (Paulsen et al. 2013). The presence of certain volatiles in the nectars of rodent pollinated plants may be related to the exploitation of a pre-existing bias (Schiestl 2005; Johnson 2006). A study looking at the social behaviour of golden hamsters *Mesocricetus auratus* found that dimethyl disulphide is a component in the urine of male mice, as well as being

present in female vaginal secretions (Singer et al. 1976; Lin et al. 2005). This supports the potential of flowers adapting to rodent attraction through the exploitation of an existing role of dimethyl disulphide in mammalian social behaviour and now in pollinator attraction. However the role of dimethyl disulphide in floral nectars of rodent-pollinated plants is still something of a mystery. It seems more likely that increased consumption of nectar with this volatile is related to its signalling of protein sources (e.g. seeds) than signalling of potential mates.

Volatiles released by the food of rodents are well documented in seed detection, where rodents use the volatile emissions from seeds as scent cues to aid in seed locating (Hollander et al. 2012; Paulsen et al. 2013). Desert hamsters (*Phodopus roborovskii*) were found to use seed volatiles – likely to be ethanol, acetic acid and sulphur compounds – to effectively locate buried seeds under laboratory conditions (Paulsen et al. 2013). Although the studies did not consider the consumption of these volatiles or test them individually, their trace presence in headspace samples from seeds suggests that rodents consume these compounds at negligible concentrations. The rejection of acetic acid and ethyl acetate in experimental diets at varying concentrations supports the prediction that rodents may be using the volatiles arising from nectar degradation as a potential indicator of food resources. Ethyl acetate in nature is an ester of ethyl alcohol and acetic acid and can only form once both are present (Reece et al. 2011), thereby the presence of ethyl acetate in floral nectars is potentially indicative of further reduction in reward quality.

As yeasts are common in a variety of floral nectars, various pollinators are known to come in contact with ethanol in their foods. Mazeh et al. (2008) conducted an experiment to identify if the presence of ethanol in food influenced the food intake of frugivorous yellow-vented bulbul (*Pycnonotus xanthopygos*). The bulbuls decreased their food intake at higher concentrations of ethanol, suggesting that the result is due to natural selection against ingestion of fruit containing potentially intoxicating levels of ethanol (Mazeh et al. 2008). Similarly a study looking at the response of frugivorous bats to varying ethanol concentrations in fruit found that bats were not attracted to high ethanol concentrations, supposedly due to the associated drop in fruit palatability and energetic value (Sanchez et al. 2006). More recently a study considering the effect of ethanol on passerine birds, white-bellied sunbirds (*Cinnyris talatala*) and Cape white-eyes (*Zosterops virens*) identified that birds show a dose-dependent response to ethanol in nectar. Both bird species tolerated ethanol

in their diets at low concentrations (0.01 and 0.03 %). However, the generalist *Z. pallidus* was found to be more tolerant to ethanol in comparison to the specialist *C. talatala* (Records 2012). This suggests that birds may use the taste of ethanol in nectar as a cue to identify nectar quality and that birds are able to tolerate low concentrations of ethanol in their food. Plants which are adapted to limit microbial activity in nectar by means of antibiotic compounds may be selected for (Records 2012). Lastly a paper by Wiens et al. (2008) found that tree shrews (*Ptilocerus lowii*), in a west Malaysian rainforest consumed ethanol at relatively high concentrations in relation to previous studies (Dudley 2002). Unlike the previously mentioned study these animals chose to consume fermented nectar and showed signs of inebriation, suggesting the potential of a coevolved relationship between ethanol consumption in tree shrews and pollination of the Bertam palm (Weins et al. 2008).

In the current study the strongest preference for volatile compounds in experimental diets was seen in *M. namaquensis*. *Micaelamys namaquensis* is a known rodent pollinator of flowers which emit distinctive yeast-like odours (*Protea amplexicaulis*, *P. humiflora* and *P. nana*), and therefore is adapted to feeding on nectar that could be degraded by some degree by microorganisms (Rourke and Wiens 1977; Wiens et al. 1983; Herrera et al. 2008; Biccard and Midgley 2009; Steenhuisen et al. 2012). These studies also record floral visitation by *R. pumilio*, which supports current findings of the increased preference seen for ethanol by *M. namaquensis* and *R. pumilio* when compared to *R. dilectus*. This is contrary to the conclusions of recent studies of third-party interactions considering insects and birds, which have described the presence of microorganisms in nectars as being disruptive to plant-pollinator mutualisms (Herrera et al. 2008; Vannette et al. 2012). Instead current findings suggest the presence of microorganisms in nectars as not being disruptive in rodent pollinated plants as they inform rodents about nectar quality.

Rodents altered their choice of experimental diet in relation to the type and concentration of volatiles in their experimental diets. As volatile concentration increased in experimental diets, preference decreased. Rodents showed the increased selection for diets that contained low concentrations of volatiles at a high sugar concentration. The relationship between preference and volatile concentration could be attributed to the rodents' use of taste to reject flowers that had their nectars degraded by microorganisms. The presence of volatile compounds arising from sugar and protein degradation in the nectars of rodent pollinated plants is potentially

indicative of previous floral visitation and therefore provides information to pollinators about the quality of nectar.

I conclude that emission of volatiles from nectar as an honest signal of nectar quality may be limited by the effects of these volatiles on nectar palatability, and that these effects are dependent on sugar concentrations in nectar. Therefore selection might favour adaptations such as secretion of compounds associated with sugar and protein degradation in the nectar of rodent-pollinated species. This may occur even if nectar is not actually degraded by micro-organisms. Investigations are needed into how alterations in floral characteristics, such as those caused by microorganisms, influence plant pollinator interactions for rodent pollinated plants. To this end, further studies of actual ethanol and other compound concentration and microorganisms in the nectar of plants are needed.

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Appendix

Table 1: Summary of various alternative statistical approaches to the analyses of the continuous proportions obtained in the rodent experimental diet consumption trials. All interactions are indicated for taste choice test experiments. Bold indicates contrasting results in the over all model for the beta distribution.

Source	Logit Transformation			Arcsine transformation			Gamma with log link			Beta distribution		
	Wald χ^2	df	Sig.	Wald χ^2	df	Sig.	Wald χ^2	df	Sig.	F Stat	df	Sig.
Species	7.145	2	<0.05	6.756	2	<0.05	6.992	2	<0.05	3.00	2	0.0505
Volatile	123.844	3	<0.001	148.527	3	<0.001	115.375	3	<0.001	31.29	3	<0.001
Volatile Concentration	631.478	2	<0.001	638.352	2	<0.001	567.995	2	<0.001	97.16	2	<0.001
Sugar Concentration	201.192	1	<0.001	205.074	1	<0.001	143.854	1	<0.001	127.79	1	<0.001
Species × Volatile × Volatile Concentration	142.386	6	<0.001	94.677	12	<0.001	170.750	12	<0.001	2.23	12	<0.01
Species × Sugar Concentration × Volatile	44.821	4	<0.001	35.409	6	<0.001	40.159	6	<0.001	0.94	6	0.4656
Species × Sugar Concentration × Volatile Concentration	11.508	2	<0.001	4.819	4	0.31	4.257	4	0.37	0.58	4	0.6758
Species × Volatile	95.237	6	<0.001	145.205	6	<0.001	99.431	6	<0.001	12.64	6	<0.001
Species × Volatile Concentration	28.869	3	<0.001	44.263	4	<0.001	47.223	4	<0.001	3.41	4	<0.01
Species × Sugar Concentration	2.703	2	0.259	18.537	2	<0.001	10.107	2	<0.01	4.73	2	<0.01
Volatile × Volatile Concentration	123.598	12	<0.001	119.664	6	<0.001	104.795	6	<0.001	12.22	6	<0.001
Sugar Concentration × Volatile	35.857	6	<0.001	36.255	3	<0.001	9.674	3	<0.05	3.19	3	<0.05
Sugar Concentration × Volatile Concentration	5.928	4	0.205	0.488	2	0.78	6.339	2	<0.05	0.46	2	0.6325
Species × Sugar Concentration × Volatile × Volatile Concentration	**139.728**	**17**	**<0.001**	**147.307**	**17**	**<0.001**	**143.781**	**17**	**<0.001**	**504**	**18**	**0.0753**

Conclusions

In this study I attempted to discover how certain volatiles associated with sugar fermentation or protein degradation may act as potential behavioural cues during rodent pollination. Two volatile compounds, ethanol and dimethyl disulphide, were highlighted as being influential to rodent pollination behaviour and the potential for them to act as behavioural cues during rodent pollination needs further attention.

The role that fermentation volatiles play in mammal pollinator-plant interactions is poorly studied. Previous studies have focused on two different aspects: on the presence of certain third party players in floral nectars, highlighting the role they play in reducing pollinator attractiveness through decreased reward quality, thus having a negative effect on plant pollinator mutualists (Madigan and Martinko 2006; Herrera et al. 2008; de Vega and Herrera 2012; Vannette et al. 2012); or alternatively the presence of certain volatiles associated with sugar and protein degradation in the foods of various animals (Steenhuisen et al. 2012a; Balmer 2013) and the effect of these compounds on animal social behaviour (Singer et al. 1976; Johnston 2003; Lin et al. 2005). Few studies have considered the potential for the volatiles associated with sugar fermentation and protein degradation to act as behavioural cues during foraging by flower-feeding rodents (Hollander et al. 2012; Paulsen et al. 2013), and no research to my knowledge has considered the potential for volatiles associated with sugar fermentation and protein degradation to influence the palatability of nectar to rodents (Table 1).

When considering rodent behavioural responses towards volatiles released by rodent pollinated plants, previously only one study has conclusively shown that a single volatile released by a plant can be attractive to rodent pollinators (Johnson et al. 2011). In chapter 2, ethanol emerged as being selected for by all three species, with the strongest response seen at the 0.3 % concentration, suggesting that concentration has an effect on rodent perception of a volatile. Studies considering the concentration of ethanol in nectar are therefore needed to better understand the relationship between rodent pollinators and this volatile.

Table 1: Rewards consumed by rodents and the potential chemical attractants exploited as indicators of potential rewards.

Rewards	Species attracted	Chemical attractant/s	References
Seeds	*Phodopus roborovskii*	Alcohols, Sulfur containing compounds and Acetic acid	Hollander et al. 2012; Paulsen et al. 2013
Nectar	*Tupaia montana* and *Rhabdomys baluensis*	Alcohols and Sulfur containing compounds	Greenwood et al. 2011; Wells et al. 2011
Nectar	*Rhabdomys pumilio* and *Elephantulus brachyrhynchus*	Aliphatic ketone	Johnson et al. 2011
Nectar and pollen	*Gerbillurus paeba, Acomys subspinosus and Micaelamys namaquensis*	Unknown	Johnson et al. 2001
Nectar and pollen	*Acomys subspinosus and Micaelamys namaquensis*	Unknown	Flemming and Nicolson 2002
Nectar and Invertebrates	*Elephantulus edwardii*	Unknown	Flemming and Nicolson 2002; 2003

Additional volatiles associated with sugar and protein degradation were tested and only *R. dilectus* responded positively to dimethyl disulphide, while acetic acid and ethyl acetate were generally not attractive to rodents (Chapter 2). Paulsen et al. (2013) found that desert hamsters (*Phodopus roborovskii*) used seed volatiles – likely ethanol, acetic acid and sulphur compounds – to effectively locate buried seeds under laboratory conditions. Therefore better data on the volatiles released by rodent pollinated flowers could assist in understanding which volatiles could be attractive to rodents and possibly give insight into how they may alter rodent behavioural responses towards resources.

The presence of volatiles associated with nectar and protein degradation in nectar alters the taste of experimental nectars, therefore having the potential to act as a pollinator cue, and may be an essential decisive factor in floral selection during pollination (Johnson et al. 2006; Zhang et al. 2012). Taste experiments revealed that both *M. namaquensis* and *R. pumilio* selected the medium (0.3 %) concentrations of ethanol in high sugar concentration diets, but found this compound distasteful in lower sugar concentration diets. Dimethyl disulphide was prefered by all species only when present in greater sugar concentrations (1.46 M) and at low volatile concentrations (0.003 %). The selection for volatile compounds at low concentrations in high sugar concentrations solutions suggest that third party interactions could be negatively influencing the fitness of plants that rely on rodent pollination; however, rodents still chose those solutions with low volatile concentrations over that of the control solutions. These findings could be important for a greater understanding of how third party interactions could potentially influence the fitness of plants that rely on rodent pollination. Further studies are needed to examine the effect of similar volatiles at various concentrations in floral nectars. These studies could determine the potential synergistic or antagonistic effects of these volatiles on rodent floral visitation.

Little is known about the effect of rodent selection for nectar with regards to monosaccharides and disaccharide sugars (Feigin et al. 1987; Johnson et al. 1999), and thus the effect that a yeast community that influences sugar composition may have on rodent visitation to flowers. More studies on how microorganisms in nectar alter sugar concentrations and proportions and the nectar chemistry of rodent

pollinated plants are needed. An example of a potential future study is the inoculation of previously sterilized protea flowers with microorganisms to establish which part of the scent comes from microorganisms and which comes from the flowers. This study could build on findings from *Silene caroliniana*, a "scentless flower", with emphasis for future investigations identifying the potential implications for alternate pollination systems (Golonka et al. 2014).

In summary, more data is needed on the assortment of volatiles emitted by rodent pollinated flowers. The little that is known about the volatiles emitted by rodent pollinated flowers has made my preliminary studies possible (Steenhuisen et al. 2012a; Balmer 2013; SD Johnson, unpublished data). Field observations and field studies are needed to examine the responses of rodents to volatiles, as these studies are essential in identifying key trends for future research in the study of the rodent pollination syndrome.

I conclude that certain compounds released through sugar fermentation or protein degradation have the potential to act as behavioural cues in rodent pollination and that further studies that reveal the effect of nectar degradation by microorganisms are needed to help in understanding plant-pollinator interactions. Emission of volatiles from nectar as an honest signal of nectar amount may be limited by the effects of these volatiles on nectar palatability, and by the dependence of these effects on sugar concentrations in nectar. The answer to the question of whether a shift from bird to rodent pollinators in the Proteaceae of the Cape mountain region could have been the result of the attraction of rodents to the smell of fermenting nectar remains elusive, but this study has shed some new light on this important question.

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