



Buzz-Pollinated Plants: Their Chemical and Behavioural Ecology

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This PhD was conducted between September 2018 – March 2023. As such, the COVID-19 Pandemic severely impacted my research through lockdowns, travel bans, and subsequent personal and societal disturbances.

I hereby declare that this dissertation is an original piece of work that embodies the results of my own research. All work contained herein has not been submitted for any other degree. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others.

A handwritten signature in black ink, appearing to read "C. Moore", with a long horizontal flourish extending to the right.

C. Douglas Moore

Abstract

This thesis explores how scent influences the attraction and behaviour of bees and, consequently, the ecology of flowers with a specialised pollination mechanism: buzz pollination. Pollen of approximately 6% of flowering plant species is contained within specialised flowers and is most effectively extracted using vibrations. These buzz-pollinated plants are primarily pollinated by bees. Floral scent is a complex phenotype widely known to mediate plant-pollinator interactions, which has recently been implicated in stimulating floral vibration production in bees visiting buzz-pollinated flowers. Yet the role of scent in regulating plant-pollinator interactions during buzz pollination is poorly understood. Firstly, I characterised how scent and sight signals of buzz-pollinated plants vary within a flower and assessed how these scent signals influenced foraging bees. Secondly, I explored how flowers with concealed pollinator rewards, for example pollen within buzz-pollinated flowers, might reliably indicate reward presence using scent. Thirdly, I investigated how scent informs decision making in pollen foraging bees. Fourthly, I explored the interactions between buzz-pollinated plants and their floral visitors in their natural environment. I found floral scent of buzz-pollinated plants to be a dynamic trait. Scent emission varied both in space and in time: between floral organs and with pollen presence, respectively. I also demonstrated that bees use scent to inform decision making when foraging for pollen. However, how bees learn to associate signals with rewards may differ when foraging for different resources, i.e., pollen and nectar. Buzzing behaviour was stimulated in bees by the complex scents produced by flowers as well as a single volatile chemical: linalool. Finally, I demonstrated that buzz-pollinated plants are regularly visited by bees which do not produce floral buzzes. These non-buzzing bees often damage flowers while collecting pollen and are expected to reduce reproductive success. Overall, these results show buzz-pollination to be a highly specialised exchange between plants and pollinators. The coordination of signals acting on multiple senses stimulates the attraction, positioning, and behaviour of bees necessary for effective pollination. Buzz-pollination has been hypothesised to select for effective pollinators, yet in natural ecosystems I found it to be regularly bypassed by pollen thieves. These findings highlight floral scent as a spatially and temporally dynamic signal used by plants to influence pollinator behaviour and learning, as well as providing novel insight into the globally important field of pollen foraging.

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Chapter One. General Introduction

The Evolution of Pollination

Sessile organisms depend on external factors to transfer genetic material for sexual reproduction. From the colonization of land by plants around 470 million years ago (Ma), early plants depended principally upon abiotic gene transfer (e.g., by wind and water); this method still persists in modern gymnosperms, clubmosses, and ferns (Taylor & Millay, 1979; Proctor *et al.*, 1996; Brown & Lemmon, 2011; Harholt *et al.*, 2016; Buschmann, 2020). However, abiotic transfer mechanisms are untargeted and susceptible to stochastic factors yielding low per-gametophyte success rates (Regal, 1977). These plants must therefore produce large numbers of male propagules, the vast majority of which fail to fertilize (Cruden, 2000), resulting in a loss of both energy and reproductive potential.

In contrast to abiotic vectors, recruiting animals to carry pollen from male to female reproductive organs (zoophily) is argued to be less wasteful and more specific (Proctor *et al.*, 1996; Ollerton & Coulthard, 2009; Ren *et al.*, 2009) and evolved independently multiple times before the rise of modern flowering plants, the angiosperms (Ren *et al.*, 2009; Cai *et al.*, 2018; Khramov & Lukashevich, 2019). The origin of angiosperms in the Early Cretaceous (Bao *et al.*, 2019) has been dated to 139 – 136 Ma by molecular phylogenies (Magallón *et al.*, 2015), a date which is corroborated by fossils of ca. 136 Ma angiosperm pollen (Crane *et al.*, 1995) and of ca. 125 Ma intact flowers (Friis *et al.*, 2006; Wang & Zheng, 2009).

Plant-pollinator interactions appear to have evolved concurrently early in angiosperm evolution (Hu *et al.*, 2008; Xiao *et al.*, 2021) and are responsible, at least in part, for the major radiations of angiosperms and pollinators during the Late Cretaceous 100 – 66 Ma (Proctor *et al.*, 1996). Indeed, the explosion of plant diversity was so rapid that Darwin dubbed it an “abominable mystery” as it appeared to contrast with the gradual change explicit in his theory of evolution by natural selection (Darwin, 1903). Today the ca. 369,000 species of flowering plants account for 96% of vascular plant diversity (Darbyshire *et al.*, 2017). Over 80% of these species (ca. 308,000) rely on animal pollination to some extent (Ollerton *et al.*, 2011; Rodger *et al.*, 2021), and one third are totally dependent on pollinators for their reproduction (Rodger *et al.*, 2021). Insects are the dominant pollinators of angiosperms, both in overall abundance and species number (Ollerton *et al.*, 2011; Aslan *et al.*, 2013), though other animals may act as pollinators, such as birds (Nelson *et al.*, 2021), bats (Fleming *et al.*, 2009), lizards (Cozien *et al.*, 2019), rodents (Johnson & Govender, 2022) and primates (Carthew & Goldingay, 1997).

The Evolution and Biology of Buzz-Pollination

Although pollination is often mutualistically beneficial for both plant and pollinator, it is also a tight balance between the often-antagonistic needs of plants and pollinators. This is especially evident when considering the fate of pollen. Plant male fitness would be optimised if all pollen sired offspring, while the nutritional requirements of pollinivorous floral visitors would be best met if they consumed all available pollen (Hargreaves *et al.*, 2009). With less than 1% of pollen removed from flowers reaching a conspecific stigma (Harder & Johnson, 2008; van der Kooi *et al.*, 2021) consumptive emasculation represents a dramatic loss of both genetic potential and energy investment, thus exerting strong selective pressure for the evolution of more efficient pollen transfer (Harder & Routley, 2006). This is reflected in the correlation between community-wide pollen limitation and angiosperm species diversity (Vamosi *et al.*, 2006) and has led to the specialisation of flowers as seen in buzz-pollinated and heterantherous plants (Vogel, 1978; Mast *et al.*, 2004).

Buzz-Pollination Modification of Plants

Buzz-pollinated flowers require vibrations to release pollen and are incidentally pollinated in the process (Michener, 1962; Vallejo-Marín, 2019). This adaptation effectively restricts pollen access to a subset of pollinators that can make these vibrations (Vallejo-Marín, 2019). Buzz-pollinated flowers have arisen independently many times and are found in at least 65 plant families (Vogel, 1978; Buchmann *et al.*, 1983; Faegri, 1986; De Luca & Vallejo-Marín, 2013), including in plants of global agricultural importance such as tomato, potato, pepper, eggplant and blueberry (Osorno-Mesa, 1947; Buchmann *et al.*, 1983; Batra, 1993; King, 1993; Morandin *et al.*, 2001; Javorek *et al.*, 2002). Pollen is typically released through pores or slits in the apex of modified anthers (i.e., poricidal anthers) which are found in ca. 6% of angiosperms or ca. 20,000 species (Buchmann *et al.*, 1983), though other tissues can form poricidal structures such as corolla tubes in some *Pedicularis* species (Corbet & Huang, 2014). Comparing different morphologies of extant flowers and the various pollen collecting strategies pollinators employ, allows us insight into how buzz-pollinated flowers may have evolved. Bees foraging on non-poricidal flowers can efficiently gather pollen by shaking it from anthers with their legs and mandibles in a behaviour called scrabbling (Thorp, 2000; Russell & Papaj, 2016). However, when resources become depleted bees can apply floral vibrations to maximise pollen collection (Russell *et al.*, 2017). This combination of behaviours is thought to have allowed poricidal plants to evolve from non-poricidal ancestors through stages of increasing concealment until the only way pollen can only be extracted is by floral vibrations (Buchmann *et al.*, 1983; Vallejo-Marín *et al.*, 2010; De Luca & Vallejo-Marín, 2013; Russell *et al.*, 2017).

Buzz-Pollinated Flowers Restrict Access to Pollinators

The ability to use vibrations to collect pollen is found in ca. 58% of all bee species, in ca. 74 bee genera (Buchmann *et al.*, 1983; Cardinal *et al.*, 2018). This behaviour has evolved independently ca. 45 times from an underlying mechanism present in a common ancestor to bees in the Early Cretaceous (Cardinal *et al.*, 2018), predating modern buzz-pollinated plant clades by at least 35 million years (Buchmann *et al.*, 1983; Friis & Crepet, 1987; Proença, 1992). Notably, this excludes honeybees (*Apis mellifera*) and, despite their ability to produce mechanistically similar behaviours, hoverflies (Rashed *et al.*, 2009; Moore & Hassall, 2016; Vallejo-Marín & Vallejo, 2021) as the single report of buzzing behaviour in *Syrphidae* remains uncorroborated (Buchmann *et al.*, 1983).

Exploration of different vibration-producing behaviours in bumblebees (i.e., flight, defensive buzzing and flower buzzing) has shown that floral buzzes are characterised by higher velocity and acceleration which may maximise pollen removal (Pritchard & Vallejo-Marín, 2020) which other pollinator clades may be unable to produce. So, poricidal anthers can act to filter potential floral visitors, in a similar manner to how long nectar tubed flowers exclude insects with short proboscises (Newman *et al.*, 2014; Kemp & Vallejo-Marín, 2021; van der Kooi *et al.*, 2021).

On The Nature of Buzzes

Exploration of the mechanics of buzz-pollination has been accelerated by drawing from the well-established fields of bioacoustics and biotremology (i.e., the study of air- and substrate-borne vibrational communication) (Gagliano *et al.*, 2012; Fletcher, 2014; Hill & Wessel, 2016; Mortimer, 2017). Floral vibrations can be broadly separated into three stages: vibration generation by bees, vibration transmission in the connected bee-flower system, and vibration-stimulated pollen release. Buzz pollination can be briefly summarised as: a bee grasping the pollen-containing structures with their legs and mandibles, curving their body over the pores, and producing vibrations (Macior, 1964; Switzer *et al.*, 2016; Russell *et al.*, 2017). Vibrations are generated through the rapid contraction of indirect flight muscles; these cause cyclical deformations in a bee's thorax which, when not flapping the wings, produces vibrations (Hedenström, 2014). Using artificially generated vibrations has shown that amplitude correlates positively with pollen release but that varying frequency in the range of natural bee buzzes (~100 – 400 Hz) has little effect (Rosi-Denadai *et al.*, 2018; De Luca *et al.*, 2019; Kemp & Vallejo-Marín, 2021; Nevard *et al.*, 2021). However, the extent to which the floral vibrations of bumblebees are adjusted, either with flower species or bee experience, is contested (Morgan *et al.*, 2016; Switzer & Combes, 2016, 2017; Russell *et al.*, 2016b; Whitehorn *et al.*, 2017; Rosi-Denadai *et al.*, 2018) and may be limited by to structural properties of the flower (Arroyo-Correa *et al.*, 2019).

Vibration transmission through the bee-flower system is achieved via physical connections of a bees' head, thorax, abdomen and legs (King, 1993; Switzer *et al.*, 2016) to the flower and is influenced by bee mass, point of attachment (Jankauski *et al.*, 2021), and properties of floral architecture and structure (Arroyo-Correa *et al.*, 2019; Brito *et al.*, 2020). The dampening or propagation of floral vibrations, and subsequent pollen release from buzz-pollinated flowers, is influenced by flower physical properties such as natural frequency, arrangement and connectivity (Figure 1) (Jankauski *et al.*, 2021; Nunes *et al.*, 2021; Nevard *et al.*, 2021; Vallejo-Marín *et al.*, 2022). Within the anther, vibrations excite the dry pollen grains and their energy increases as they impact the internal anther structures before ejection from the pores (King & Lengoc, 1993; Hansen *et al.*, 2021; Jankauski *et al.*, 2022) with the amount of pollen released affected by pore aperture and amount of pollen available (Harder & Barclay, 1994; Kemp & Vallejo-Marín, 2021). Finally, the release of pollen from buzz-pollinated morphologies is staggered by two mechanisms which are expected to result in a greater number of bee visits through the life cycle of a flower. At short timescales (seconds to minutes) multiple buzzes are required to release all pollen available in the flower (the equivalent of 3-10 seconds of continuous buzzing), while sequential pollen maturation by flowers spreads pollen release over longer timeframes (hours and days) (Corbet *et al.*, 1988; King & Buchmann, 1996; Kemp & Vallejo-Marín, 2021).

Ecology of Buzz-Pollination

Despite recent interest in buzz-pollination, few naturalistic studies have been undertaken to place theoretical and experimental findings into ecologically relevant plant-pollinator networks *in situ* (Machado & Lopes, 2004; Mesquita-Neto *et al.*, 2018). The field studies that have been conducted show that buzz pollinated plants are commonly visited by inefficient pollinators such as non-buzzing insects or buzzing bees which, due to morphological mismatch, do not deposit pollen on stigmas (Solís-Montero & Vallejo-Marín, 2017; Vega-Polanco *et al.*, 2020; Mesquita-Neto *et al.*, 2021). Buzz-pollinated plants are particularly well suited to comparing the impact of mutualistic and non-mutualistic floral visitors on pollinator networks due to: a distinctive audible component of flower buzzing, their generally conspicuous stigmas, and the reduced number of pollinator groups visiting these plants (especially of nocturnal lepidopterans which are generally missed by predominantly diurnal flower observations; Van Zandt *et al.*, 2020).



Figure 1: various anther arrangements in poricidal buzz-pollinated flowers. Anthers a may form cones by anther wall fusion (A; *Echeandia luteola*) or adhesive secretions in (B; *Solanum dulcamara*), be held in loose arrangements (C; *Solanum elaeagnifolium*) and present heteranthery (D; *Solanum rostratum*).

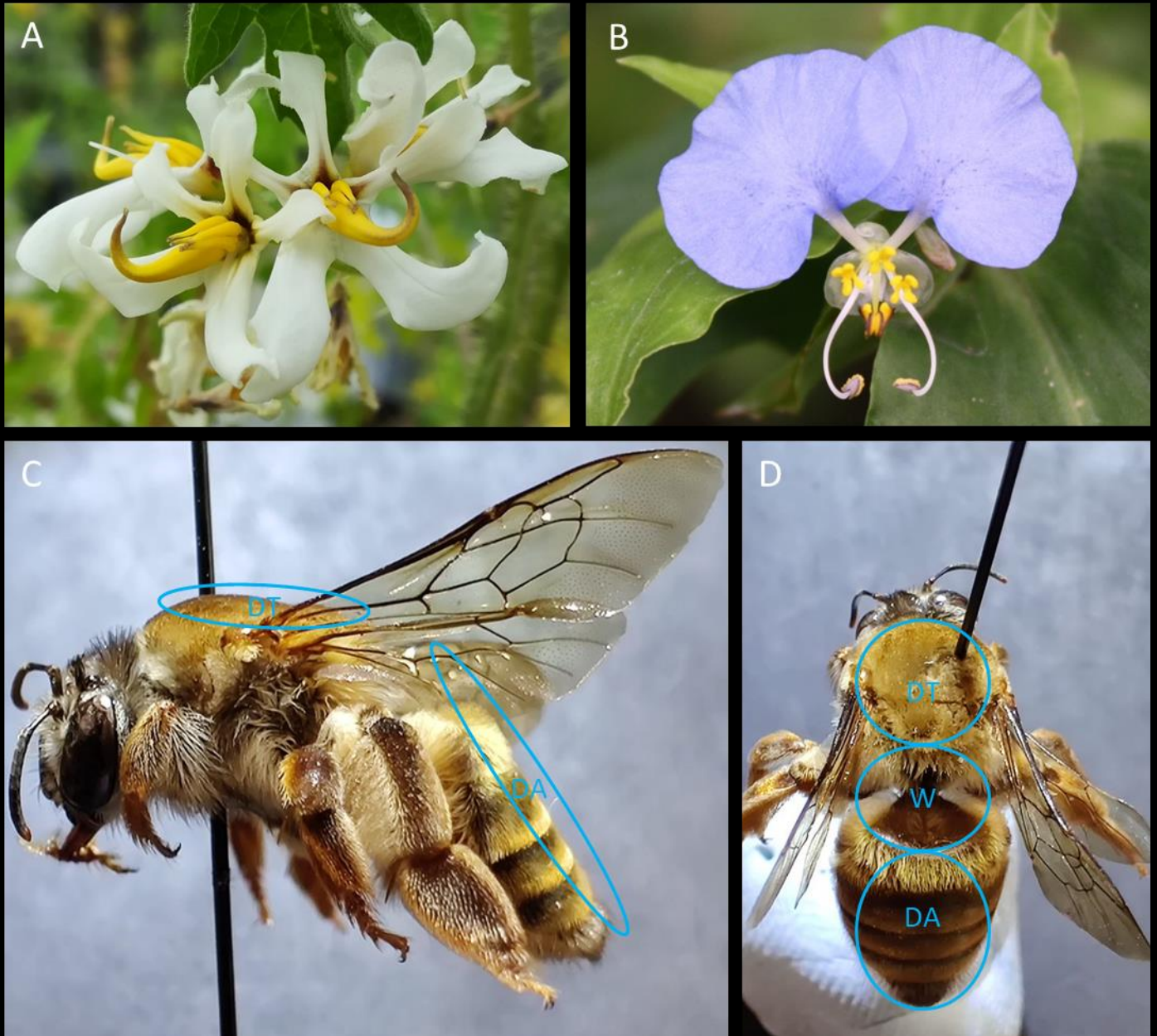


Figure 2. Heteranthery, where two or more distinct anther morphotypes exist in a single flower, has evolved in angiosperms multiple times, and occurs in at least 20 families across among 12 orders. This trait is often associated with buzz-pollinated plants such as *Solanum grayi grandiflorum* (A) but not exclusively so, as demonstrated by *Commelina erecta* (B). Both species demonstrate the most common form of heteranthery, anther dimorphism, where one anther morph is typically centrally located, conspicuously coloured (i.e., feeding anthers), and the second anther morph is often larger, located away from the centre of the flower and less visually conspicuous (i.e., pollinating anthers). It is generally accepted that the two anther morphologies evolve under divergent selection pressures causing specialisation to the role of pollinator nutrition in the feeding anthers, and to reproduction in the pollinating anthers. Thus, allowing plants to regulate how much pollen is invested to these two functions. Reproductive success is improved by pollinating anthers depositing pollen on hard to groom “safe sites” (C, D) such as bees’ dorsal thorax (DT), waist (W) and dorsal abdomen (DA).

Heteranthery

Heteranthery, the presence of two or more morphologically distinct anthers in the same flower (Figure 2), is an adaptation which has evolved in plants many times to improve pollen transfer efficiency and is often associated with buzz-pollination (Luo *et al.*, 2008; Vallejo-Marín *et al.*, 2010; Barrett, 2021; Trevizan *et al.*, 2023). The typical C-shaped posture adopted by bees when producing floral vibrations (De Luca & Vallejo-Marín, 2013) aids collection of pollen (Vallejo-Marín *et al.*, 2009; Koch *et al.*, 2017; Tong *et al.*, 2019) which is lost for the purposes of pollination once packed in bees' corbiculae (Parker *et al.*, 2015; Russell *et al.*, 2021). Deposition of pollen on different sites of a bees' body, as occurs in buzz-pollinated flowers with loosely held stamens (Figure 1) (Vallejo-Marín *et al.*, 2022), can take advantage of hard-to-groom "safe sites" (Figure 2). This can result in increased successful pollen export to conspecific stigmas and siring success (Koch *et al.*, 2017; Tong & Huang, 2018), so producing a pressure to evolve specialised anthers (Vallejo-Marín *et al.*, 2010, 2022; Barrett, 2021). The division of labour in heterantherous flowers is achieved by (two or more) morphologically distinct stamen types placing pollen on (1) easy-to-groom sites to feed pollinators (feeding anthers), and (2) harder to groom sites more likely to make contact with stigmas (pollinating anthers) (Figure 2) (Luo *et al.*, 2008; Solís-Montero *et al.*, 2018; Velloso *et al.*, 2018). By uncoupling these functions, divergent selection pressures produce anthers that differ in colour, position, size, shape and scent (Vallejo-Marín *et al.*, 2014, 2022; Solís-Montero *et al.*, 2018; Dellinger *et al.*, 2019; Valadão-Mendes *et al.*, 2022; Basso-Alves *et al.*, 2022; Trevizan *et al.*, 2023). Indeed, in some cases pollen of the feeding anthers, which is most likely to be consumed, receives less investment from plants, causing reduced fertility and cytoplasmic content (Nepi *et al.*, 2003; Luo *et al.*, 2009; Pinheiro-Costa *et al.*, 2018). The common association of heteranthery with buzz-pollination, and the loss of nectar, suggests these traits function together to increase cross pollination (Barrett, 2021). Yet, despite the proposed impact of these adaptations on plant-pollinator communication, the effect of heteranthery on pollinator behaviour has been little investigated experimentally (Li *et al.*, 2015; Papaj *et al.*, 2017).

Plant-Pollinator Communication

Floral Scent & Chemical Ecology

Flowering plants use a diverse array of signals to communicate with pollinators including sight (Lunau *et al.*, 2021), smell (Ramirez *et al.*, 2023), heat (Harrap *et al.*, 2017), static electricity (Clarke *et al.*, 2013), humidity (Harrap *et al.*, 2021) and acoustics (Simon *et al.*, 2011, 2023) (reviewed in Raguso, 2004). Here, we define a "signal" as any evolved means to transmit information actively from an organism, the emitter, to influence the behaviour of another organism, the receiver (e.g.,

scented secondary metabolites added to nectar to attract pollinators; Burdon *et al.*, 2020). In contrast, a cue passively transmits information from an emitter to a receiver but is produced by the emitter as an artefact of another biological function (e.g., pollinators can use floral humidity, largely produced by evaporation of nectar, to select profitable flowers; von Arx *et al.*, 2012).

The seminal work of von Frisch demonstrated that bees use combinations of sensory modes to inform foraging decisions (von Frisch, 1954, 1971), with sight and smell dominating. Indeed, the fact that insects can smell and that smell plays a key role in pollination has been known for millennia (Aristotle, *The History of Animals*, ca. 350 BCE, translation by Cresswell (1862). Yet scent as a plant-pollinator signal remains relatively under-researched (Raguso, 2008a), likely due to the role of vision as the dominant sense in humans, the technical difficulty of studying scent, and a lack of sufficient collaboration between relevant disciplines (Raguso, 2008b; Raguso *et al.*, 2015; Byers, 2021a). However, the last two decades has seen an acceleration in the study of floral scents, due to the decreased cost of gas chromatography-mass spectrometry (a technology central to the field used to separate and identify chemical compounds), and increasing access to key analytical services (Byers, 2021a). This has led to a rapid expansion of our understanding of scent in plant-pollinator communication (although many barriers remain, see Raguso, 2008a,b; Bohman *et al.*, 2016, 2020; Byers, 2021), revealing that scent is a dynamic and complex trait in many aspects.

Over 2,000 volatile organic compounds (VOCs) have been identified in floral scents (Knudsen *et al.*, 2006; El-Sayed, 2019), with a single species emitting potentially hundreds of compounds (Gfrerer *et al.*, 2021) though 20-60 is more common (Knudsen & Gershenzon, 2020). Within this abundance certain compounds are over-represented, such as the monoterpenes linalool and limonene which are found in over 54% of plant families, while others may be only produced by a single species (Knudsen *et al.*, 2006). The function of VOCs can range from broad to specific. Linalool is a VOC with broad function which acts as a generalist pollinator attractant (Raguso & Pichersky, 1999; Raguso, 2016), inhibits pest oviposition (De Moraes *et al.*, 2001) and attracts natural enemies (Kessler & Baldwin, 2001). In contrast VOC function can be very specific, as seen in the floral displays of *Chiloglottis trapeziformis*. Here, the orchid replicates the mating displays of sexually active female *Neozeleboria* wasps by producing a wasp pheromone, 2-ethyl-5-propyl cyclohexan-1,3-dione, as well as visual and tactile stimuli. Deceived male wasps copulate with the orchid's flowers which deposit or receive pollinaria from the insect, thus securing pollination via sexual deception (Schiestl *et al.*, 2003; Raguso, 2004a; Byers, 2021b).

Complexity of Floral Scent

Both the presence and relative abundance of the chemicals in scent are important for foraging insects, with relatively minor scent components playing a vital role (Dobson *et al.*, 2005; Zhao *et al.*, 2022). For example, methyleugenol is highly attractive to pollinators such as bees and flies, but proves repellent to herbivores when present in high concentrations (Dobson, 1994; Tan & Nishida, 2012). Likewise, α -Copaene is highly attractive to flies at low concentrations but high concentrations cause abnormal behaviour (Nishida *et al.*, 2000). Thus, the diversity and abundance of floral VOCs allows plants to do many things: to communicate with specific pollinators, to influence pollinator learning, and to trigger insect behavioural responses such as copulation in sexual mimicry and vibrations in buzz-pollination (Wright & Schiestl, 2009; Schiestl, 2010a; Russell *et al.*, 2018; Byers, 2021b).

Floral Scent as a Dynamic Trait: Spatial Variation

For over 60 years we have known that floral scent is a variable trait (Overland, 1960), yet only relatively recent technical developments (as in Tholl *et al.*, 2006) have begun to reveal just how dynamic floral scent can be, allowing plants acute control of signal transmission (Burdon *et al.*, 2015, 2020; García *et al.*, 2021). Studying where and when volatile compounds are emitted provides insight into their ecological function (Muhlemann *et al.*, 2014), and has revealed that floral scents have functions beyond pollination such as defence against predators (Scala *et al.*, 2013), parasites (Morinaga *et al.*, 2009) and pathogens (Huang *et al.*, 2012).

Experimental studies have shown that floral scents are composed of VOCs emitted by different organs which are active across spatial scales from kilometres to millimetres (Knudsen *et al.*, 1999; Dötterl & Jürgens, 2005) and regulate various aspects of plant-pollinator interactions (Russell *et al.*, 2018; García *et al.*, 2021). Airborne scent plumes are commonly emitted by anthers and petals, allowing long range attraction of pollinators beyond the line-of-sight, sometimes operating at the kilometre scale (Ackerman, 1981; Dobson *et al.*, 2005). At shorter ranges, pollinators can use scent variation to make foraging decisions and select the most rewarding flowers in a community, patch or on an individual plant (Raguso, 2004b; Burdon *et al.*, 2020). At the finest scale, scent guides on petals orient pollinators on the flower (Dötterl & Jürgens, 2005), and pollen and nectar often emit VOCs to attract pollinators directly (Dobson *et al.*, 1999; Raguso, 2004b; Burdon *et al.*, 2020).

Floral Scent as a Dynamic Trait: Temporal Variation

Temporal variation in scent emission allows plants to adapt to changing biotic and abiotic factors. Seasonal variation in VOC has been recorded at the community level, with plants flowering when

pollinators are scarce early in the season producing stronger scents than plants which flower when pollinators are abundant (Filella *et al.*, 2013). Environmental factors such as drought can also precipitate community level changes to VOC emission which consequences for reproductive success (Jaworski *et al.*, 2022; Castro *et al.*, 2023). Many plant species also exhibit daily cycles of scent emission, synchronising signalling effort with the activity of key pollinators (Raguso & Willis, 2005; Morinaga *et al.*, 2009; Balao *et al.*, 2011). VOC emissions can also change through the lifecycle of a flower: male-phase flowers of the protandrous *Aconitum napellus* produce toxic compounds to prevent pollen consumption but compensate for potentially reduced pollinator visitation by emitting more attractive VOCs than female-phase flowers (Jacquemart *et al.*, 2019). Furthermore, permanent scent changes occur in response to herbivory (Kessler *et al.*, 2011), florivory (Vega-Polanco *et al.*, 2020) and pollination (Schiestl *et al.*, 1997; Theis & Raguso, 2005; Burdon *et al.*, 2015). Overall, floral scent appears to be a highly labile trait susceptible to selection (Parachnowitsch *et al.*, 2012) which can produce shifts in scent emission across a species' range (Chapurlat *et al.*, 2018; Mackin *et al.*, 2021).

Scent Variation with Reward Availability

Floral scents may also vary with reward availability, with emissions rising and falling as rewards are depleted and replenished (Pacini & Hesse, 2005; Wright & Schiestl, 2009; Burdon *et al.*, 2015, 2020). Signals which correlate with reward availability or quality can be considered “honest” as they provide accurate information about rewards. Alternatively, “dishonest” signals advertise the presence of rewards or resources which are not present (e.g., constitutively unrewarding flowers such as brood site and food deception; Raguso, 2004a; Schiestl, 2005) while signals may be non-honest if they do not correlate with rewards (e.g., floral colour is usually an unvarying signal which indicates reward location without providing information on reward status). Indeed, emission of some scent signals may be constrained by the biosynthetic pathways they share with visual signals and vice versa. For example, the terpenes are a class of organic compounds which includes scent (e.g., linalool, geraniol and limonene) and pigments (e.g., beta-carotene, lycopene and xanthophyll) involved in floral signalling (Yeon & Kim, 2021). Pollinators are expected to prefer “honest signals” as they should allow selection of the most rewarding flowers and facilitate optimal foraging (Wright & Schiestl, 2009). Indeed, fossil evidence suggests that the rewards of primitive flowers emitted visual and olfactory signals (Crepet, 1983), but in modern flowers signalling is typically done by non-rewarding tissues (Raguso, 2008a; Leonard *et al.*, 2011b). This suggests that production of scented rewards is either unfeasible or not beneficial, perhaps because small quantities of rewards are unable to emit signals of sufficient strength to attract pollinators, or perhaps because the addition of secondary metabolites renders rewards distasteful or toxic (Stevenson *et al.*, 2017; Rivest & Forrest,

2019). Alternatively, the evolution of floral structures which protect pollen against biotic and abiotic stressors, such as desiccation, ultraviolet light, and undesirable floral visitors (Buchmann *et al.*, 1983; Bock *et al.*, 1989; Gao *et al.*, 2019), may restrict or prevent signal transmission from rewards. In cases where rewards are concealed, pollinators must rely on signals from non-rewarding floral tissues, which means that plants could exploit pollinators by signalling dishonestly to maximise pollen transfer. A combination of observational and experimental research is needed to begin establishing how pollinators disentangle the complex variety of floral cues they encounter in order to optimise foraging efficiency.

Thesis Aims and Structure

In this thesis, I focus on exploring the chemical ecology of buzz-pollinated flowers and the influence of floral scent on pollinator behaviour. I combine both experimental and empirical approaches, with emphasis on spatio-temporal scent variation. The overall aims of my thesis were to:

- a) characterise the visual and chemical signals produced by the organs of heterantherous buzz-pollinated flowers and assay the impact of scent on bee foraging behaviour; expecting that another division of labour extends to olfaction (Chapter Two).
- b) determine whether flowers with concealed floral rewards, namely pollen in buzz-pollinated flowers, produce olfactory signals which fluctuate with reward availability; hypothesising that pollen removal would result in significant changes in floral scent emission (Chapter Three).
- c) investigate how bees use scent to inform decisions when foraging for pollen; predicting that bees will use scent to influence initial floral choice but learn to overcome initial preferences which prove unrewarding (Chapter Four).
- d) explore the composition of natural pollination networks of buzz pollinated plants; hypothesising that network structure will differ between sites and also when visits from legitimate (buzzing) and illegitimate (non-buzzing) floral visitors are considered (Chapter Five).

Chapter Two. Pollinator Responses to the Scent of Buzz-Pollinated Flowers with Morphologically Distinct Anthers

Floral scent varies spatially, with distinct organs contributing different compounds to the overall signalling effort which influences the attraction of pollinators (Raguso, 2004a) and triggering the production of floral vibrations in bees visiting buzz-pollinated flowers (Russell *et al.*, 2018). Many buzz-pollinated flowers are heterantherous (Vallejo-Marín *et al.*, 2010, 2014) yet how the various aspects of plant-pollinator signalling are divided across heterantherous buzz-pollinated flowers is poorly understood. In this chapter I describe the visual and olfactory signals produced by different parts of a heterantherous, buzz-pollinated flower (*Solanum rostratum*) using spectrophotometry and

gas chromatography-mass spectrometry, respectively. I also explore the impact of olfactory cues from different floral parts on the behaviour of a model pollinator (*Bombus terrestris*).

I asked the following questions: (1) What colour differences (i.e., spectral) exist between floral parts of *Solanum rostratum*, and (2) how do bees' visual systems perceive differences? (3) How do scents produced by feeding anthers, pollinating anthers, and corollas influence bee attraction and foraging behaviour? And (4), which VOCs are emitted by different floral parts?

Chapter Three: Pollen Removal Changes Floral Scent of Buzz-Pollinated *Solanum* Flowers

Floral signals which fluctuate through time allow dynamic plant-pollinator communication. For example, signals which correlate with reward availability inform pollinator decision making, as has been demonstrated for nectar (Knauer & Schiestl, 2015; Burdon *et al.*, 2020). Yet, despite many buzz-pollinated flowers being nectarless and their pollen being concealed within floral structures that prevent direct observation, foraging insects can make accurate foraging decisions (Buchmann & Cane, 1989). Therefore, I hypothesised that floral scent emission will fluctuate with pollen availability. Using gas chromatography-mass spectrometry, I investigate whether scent composition and emission rate vary with pollen availability by comparing the scents produced by flowers with and without pollen. I ask the following questions: (1) Does the volume of floral scent vary with pollen presence and (2) Does the composition of floral scent vary with pollen presence?

Chapter Four: Use of Scent Signals by Pollen Foraging Bees

In this chapter, I explore how scent influences the preference, behaviour and learning of pollen foraging insects. All flowering plants produce pollen. It is a rich source of proteins and lipids that is collected by floral visitors for direct consumption or to feed to offspring. Indeed, in over 10% of angiosperms, including many buzz-pollinated species, pollen is the only reward produced. Scent is a key component of floral signalling, which informs pollinator foraging decision making and triggers specialist pollen collecting behaviours (i.e., buzz pollination). However, little is known about how pollinators use scent to make decisions when foraging for pollen. I asked the following questions: (1) does scent influence initial flower selection of pollen foraging bees, and (2) do bees learn to overcome initial scent preferences which prove unrewarding? I observed bumblebee' choices and behaviours when foraging for an extended time on an array containing two types of artificial flowers in which scent and a pollen surrogate were either paired or unpaired.

Chapter Five: Pollination Networks of Plants with Buzz-Pollinated Flowers in a Tropical Dry Forest Community (Yucatán, Mexico)

In this chapter I explored the interactions between buzz-pollinated plants and their floral visitors in five natural communities in the field. Less than 1% of pollen reaches conspecific stigmas (Harder & Johnson, 2008); pollen limitation often exerts strong selective pressure for the evolution of more efficient pollen transfer (Harder & Routley, 2006), which is reflected in the geographic correlation between pollen limitation and angiosperm species diversity (Vamosi *et al.*, 2006). Pollen loss to inefficient pollinators is theorised to be the driving pressure behind the evolution of buzz-pollinated flowers, restricting access to bees capable of vibrating flowers (Buchmann *et al.*, 1983). Yet despite the protection offered by enclosing pollen within specialised floral morphologies, buzz-pollinated flowers are regularly visited by illegitimate visitors which rob pollen (Mesquita-Neto *et al.*, 2018). I used focal plant observations to construct pollination networks, classify floral visitors as either mutualists or thieves, and identify them to morphospecies. I asked the following questions: (1) how do plant-pollinator interaction networks vary between sites, (2) to what degree do floral visitors specialise to buzz-pollinated plant species, (3) does the functional role of floral visitors (i.e., mutualist or thief) vary between plant species and, (4) how do pollination networks differ if we consider all floral visitors or only mutualists?

Chapter Six: General Discussion

In this final chapter, I discuss the findings from my four data chapters in the context of the broader literature, specifically addressing:

1. Spatial variation of scent emission in buzz-pollinated plants.
2. Temporal variation of scent in buzz-pollinated plants in relation to pollen availability.
3. Whether division of labour in heterantherous plants extends to scent.
4. The use of floral scent by pollen foraging bees.
5. Plant-pollinator interactions of buzz pollinated plants.

Chapter Two. Pollinator Responses to the Scent of Buzz-Pollinated Flowers with Morphologically Distinct Anthers

Abstract

Scent is often a key component of floral displays and can influence pollinator behaviour. For example, scent appears a key trigger for the production of thoracic vibrations in bees collecting pollen from certain flowers. Buzz-pollinated plants depend on these vibrations for pollen release. This is occasionally accompanied by heteranthery, whereby two or more anther types are present in the same flower, specialising to the roles of rewarding bees (feeding anthers) and plant fertilization (pollinating anthers). Yet how different organs of buzz-pollinated flowers contribute to pollinator attraction and behavioural manipulation is poorly understood. Here we investigated how scent variation between organs of buzz-pollinated flowers influenced pollinator attraction and behaviour. We compared the choices and behaviours of free-flying bumblebees (*Bombus terrestris*) foraging for pollen on artificial flower arrays treated with scent extracts from different floral organs of the buzz-pollinated and heterantherous *Solanum rostratum*. We also quantitatively described the olfactory and visual signals produced by different floral organs. Androecium scent was more attractive to bees than corolla scent, stimulating longer floral visits and more pollen collecting behaviour. Within the androecium there was no difference in bee preference or the likelihood of bees buzzing. Floral organs should appear visually distinct to bees, and their scent differed in overall volume, presence, and abundance of chemicals emitted. These results show that the floral organs of heterantherous plants may not be solely specialised to either the role of pollinator feeding or plant reproduction but can produce signals performing both specialist and generalized plant-pollinator communication functions but suggest that the specialist and generalist roles must be performed in discrete, non-overlapping communication channels.

Keywords

Buzz-pollination, *Solanum*, heteranthery, pollinator, olfaction, vision, chemical ecology, bee behaviour, *Bombus terrestris*, pollen foraging, flower signalling.

Introduction

Flowers are sensory billboards through which plants communicate with their pollinators (Raguso, 2004a). Floral displays can involve an array of cues using visual, olfactory, and tactile components as well as static electricity and humidity (Raguso, 2004a; Tholl *et al.*, 2006; von Arx *et al.*, 2012). Scent is often a key component of floral displays, consisting of volatile organic compounds (VOCs), >2000 of which have been described (Knudsen *et al.*, 2006; El-Sayed, 2019). Floral scent consist of mixtures of these VOCs, up to 200 of which may be present in a single species (Gfrerer *et al.*, 2021). Pollinators are sensitive to both the presence and relative abundance of VOCs in a scent blend, using both to inform foraging decisions (Byers *et al.*, 2014; Zhao *et al.*, 2022). Plants exploit this sensitivity to attract targeted pollinator species, affecting their learning, and eliciting specific behaviours (Wright & Schiestl, 2009; Schiestl, 2010a; Russell *et al.*, 2018).

The overall scent of a flower may consist of groups of compounds produced by different organs and tissues with specialised roles to control diverse aspects of plant-pollinator interactions (Russell *et al.*, 2018; García *et al.*, 2021). For example, osmophores on anthers and petals produce airborne scent plumes which can attract pollinators beyond the line-of-sight and at ranges greater than a kilometre (Ackerman, 1981; Dobson *et al.*, 2005). At short range, scent variation allows pollinators to select the most rewarding flowers within a community, patch or on an individual plant (Chapter Three) (Raguso, 2004b; Burdon *et al.*, 2020). Within the flower, scent guides on petals direct pollinators towards the centre of flowers (Dötterl & Jürgens, 2005) or to where rewards are located, and rewards themselves are often scented (e.g., nectar and pollen) (Dobson *et al.*, 1999; Raguso, 2004b; Burdon *et al.*, 2020), directly attracting pollinators.

In addition to attracting and directing pollinators, floral scent is also implicated in eliciting complex, derived or unusual behaviours in pollinators. For example, the production of thoracic vibrations by pollen collecting bees in certain flowers (Russell *et al.*, 2017, 2018). Flowers which rely on these vibrations for pollen release and thus pollen transfer are said to be buzz-pollinated, often exhibiting a shared morphology where pollen is expelled from the flower through small pores or slits (i.e., poricidal flowers), in contrast to more common laterally dehiscent anthers (Buchmann *et al.*, 1983; De Luca & Vallejo-Marín, 2013). This effectively restricts pollen access to approximately 70 bee genera, representing ca. 58% of bee species (Hedtke *et al.*, 2013; Ascher & Pickering, 2020), which are capable of producing the necessary vibrations. If scent is a necessary trigger for bee buzzes on buzz-pollinated flowers (Russell *et al.*, 2017, 2018) then it should be widespread and the compounds responsible may be isolated.

Another mechanism whereby plants reduce pollen loss and improve pollen transfer to conspecifics is heteranthery, where flowers possess two or more distinct anther morphs (Luo *et al.*, 2008; Solís-Montero *et al.*, 2018; Velloso *et al.*, 2018; Barrett, 2021). The Division of Labour Hypothesis has been proposed as an explanation for these different anther morphs, whereby pollen produced by one anther type is destined to reward pollinators (i.e., feeding anthers) while another for pollen export and plant fertilization (i.e., pollinating anthers) (Luo *et al.*, 2008; Vallejo-Marín *et al.*, 2009). The separation of roles results in divergent selection producing anther morphs which differ in various traits including colour, position, arrangement, and scent (Vallejo-Marín *et al.*, 2014; Solís-Montero *et al.*, 2018; Dellinger *et al.*, 2019; Valadão-Mendes *et al.*, 2022; Basso-Alves *et al.*, 2022). Feeding anthers are typically easy for pollinators to encounter: they occupy a central position in the flower and are more conspicuous to pollinators (e.g. in colour and scent). Meanwhile, pollinating anthers are less conspicuous and often centrally displaced, appearing to be positioned to place pollen on safe sites of a bees' body which cannot be groomed easily and where conspecific stigmas are best positioned to collect pollen from (Koch *et al.*, 2017; Tong & Huang, 2018). This specialisation is sometimes reflected in pollen investment: pollen of pollinating anthers is "normal" while that of feeding anthers receives lower genetic and energetic investment resulting in low fertility and low cytoplasmic content (Nepi *et al.*, 2003; Luo *et al.*, 2009; Pinheiro-Costa *et al.*, 2018). The specialisation of heteranthery extends to pollinator communication, with feeding anthers being more attractive (Solís-Montero *et al.*, 2018; Basso-Alves *et al.*, 2022). However, the effect of heteranthery on pollinator behaviour has been little investigated.

In this study, we sought to characterise how scent variation between the organs of buzz-pollinated flowers (corolla, feeding anthers and pollinating anthers) influences pollinator attraction and behaviour. We conducted a pair of laboratory experiments using *Solanum rostratum* (Dunal), a heterantherous species which produces no nectar, and the generalist pollinator, *Bombus terrestris* (Linnaeus). We further characterised the spectral and chemical signals of floral organs. We expected bees to prefer scents of floral origin over controls, with anthers more attractive than corollas (Russell *et al.*, 2017, 2018; Solís-Montero *et al.*, 2018). We hypothesised that anther morphs differ both in their attraction and in their capacity to stimulate buzzing pollen collection behaviour of bees. We therefore expect buzzing to be elicited by scents extracted from androecium and not by the corolla (Russell *et al.*, 2017, 2018). Furthermore, we hypothesised that heteranthery extends to chemical signals and so expected bees to be more attracted to and more likely to buzz on extracts of feeding anthers over pollinating anthers. Flowers were equally rewarding so we did not expect bee preference to change through time.

Methods

Study Outline

To assess the influence of scent from different organs of buzz-pollinated flowers on pollinator foraging preferences and behaviours we observed bumblebees foraging for a pollen surrogate on artificial flower arrays. Two experiments were conducted, presenting three scent conditions simultaneously, two from floral organs of the buzz-pollinated herb, *S. rostratum*, and a control. In Experiment 1: Androecium Vs Corolla, bees foraged on flowers scented with either corolla extract, androecium extract, or control. In Experiment 2: Feeding vs Pollinating Anther, bees foraged on flowers scented with either feeding anther extract, pollinating anther extract, or control. Hexane was the solvent used to produce floral scent extracts and was used as the negative control. Individual bees foraged freely for up to 50 floral visits in a single bout and were allowed multiple foraging bouts (Figure 3). Bees' initial and changing scent preferences were assessed by recording which flowers were visited. Experiments took place at the University of Stirling between September-December 2020 and September-October 2021.

Bees

Commercially obtained *Bombus terrestris audax* workers (Biobest Group NV, Westerlo, Belgium) were used to conduct laboratory experiments. Bumblebees are generalist pollinators which forage on many hundreds of angiosperm species across their global range (Plowright & Lavery, 1984) and can adapt to completely novel flora (Dafni *et al.*, 2010). Colonies were maintained at 18-25°C, and fed *ad libitum* unscented Biogluc sucrose solution (Biobest Group NV, Westerlo, Belgium) and pulverised honeybee-collected pollen from the UK (Agralan Ltd., Wiltshire, UK).

Plant Material

Solanum rostratum plants were grown at the University of Stirling from seeds collected from wild populations in Mexico in 2010 (Table 1) (Vallejo-Marín *et al.*, 2014; Nunes *et al.*, 2021). To facilitate germination, seeds were soaked for 24 hours in 1,000 ppm aqueous gibberellic acid solution (GA3; Sigma-Aldrich, Dorset, UK) at room temperature. Seeds were then individually planted in 20cm³ seed trays (2×2×5cm) of modular seed growing medium compost (William Sinclair Horticulture PLC, Lincoln, UK), placed in a growth chamber and maintained at 28.4°C ($\pm 2.6\sigma$), and 52.4% relative humidity ($\sigma = \pm 5.6$), illuminated at 4.8-9.5klx in an 18:6 Light:Dark cycle for 2-4 weeks. Seedlings were then transferred to individual 1.5L pots (15cm diameter) containing a 4:1 mix of All Purpose Growing Medium and Perlite Standard (William Sinclair Horticulture PLC, Lincoln, UK) and placed in a pollinator-proofed greenhouse, lit by sunlight supplemented with compact-florescent lights to

ensure ≥ 16 hours daily illumination. Plants were watered 3 times a week, as needed, fertilized weekly with Tomorite Concentrated Tomato Food (Levington, Surrey, UK) diluted to manufacturer's instruction, and maintained between 16-25°C.

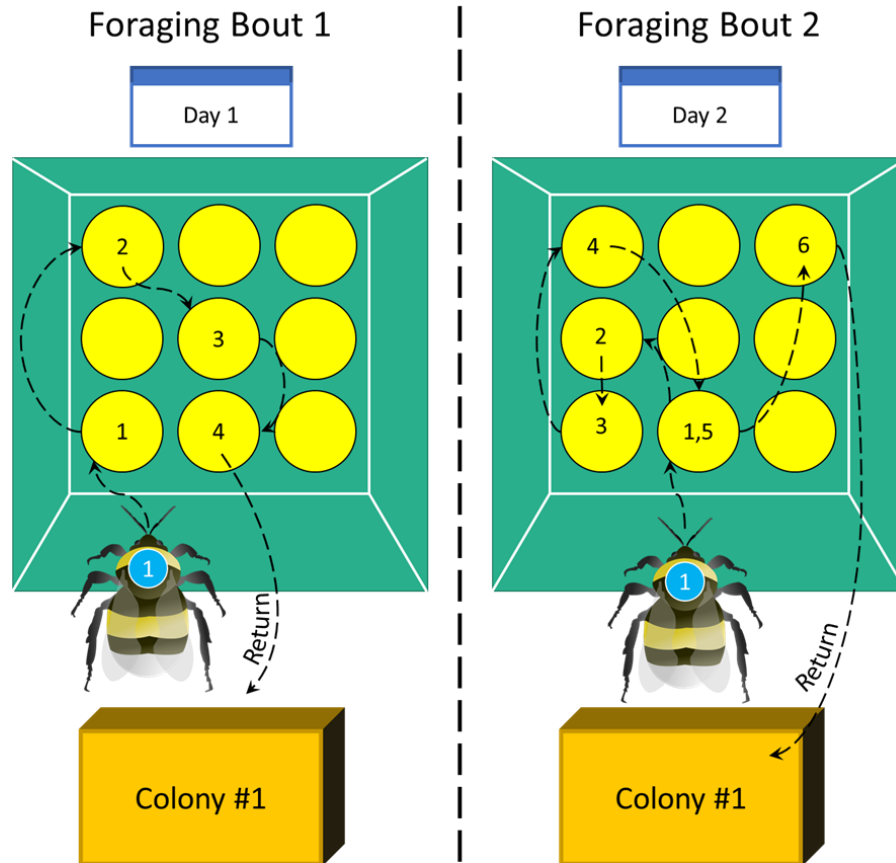


Figure 3. Bees were observed foraging on an array of visually identical artificial flowers. Three flowers of each scent treatment were presented in a 3×3 vertical grid on the flight arena wall. A floral visit was defined as a bee deliberately contacting a flower in the array. The continuous time a bee spent in the experimental arena was classified as a foraging bout. Bees were allowed to undertake two foraging bouts on separate days. Here, the bee Blue1 from Colony1 visits four flowers in its first foraging bout before returning to the colony. The next day Blue1 makes a second foraging bout making six floral visits, two of which (1 and 5) are to the same flower.

Floral Extract Preparation

Undamaged virgin *Solanum rostratum* flowers were collected, within 24 hours of opening. Flowers were separated from vegetative material by firmly pinching the calyx while gently pulling the corolla, and anthers were then removed by snapping the filament. Floral organs from four flowers were separated (feeding anthers, pollinating anthers, androecium or corollas, see Figure 5) and

submerged in a 15ml glass phial containing 4ml hexane (95% purity, Sigma-Aldrich) for 5 minutes. Floral organs were removed from the solvent using clean forceps and as much hexane as possible shaken off, the volume of hexane topped up to 4ml and the process repeated until the solution contained 128 flowers (32 flowers ml⁻¹) equivalent scent extract. Sealed phials were stored at -18°C until use.

Table 1: *Solanum rostratum* seeds collected from a wild Mexican population in 2010 (Vallejo-Marín et al., 2014).

Accession	Location	Latitude (N)	Longitude (E)	Elevation (m)
10 s 71	San Miguel de Allende, Queretaro State, Mexico	20.90185	100.70547	2,033
10 s 72	San Miguel de Allende, Queretaro State, Mexico	20.90185	100.70547	2,033
10 s 74	San Miguel de Allende, Queretaro State, Mexico	20.90185	100.70547	2,033



Figure 4. Experimental flowers consisted of a yellow cardboard corolla and a white pipe cleaner anther containing the pollen surrogate. Here a bumblebee forager grooms the pollen surrogate from her body which has been removed from the artificial flower.

Flight Arena And Artificial Flowers

The experimental arena (LWH: 70×50×25 cm) consisted of wooden floor and walls painted grey, a clear acrylic ceiling and clear PVC access panel. Lighting was provided by an LED light panel (59.5 × 59.5 cm, 48W Daylight; Opus Lighting Technology, Birmingham, UK). Artificial flowers consisted of a yellow cardboard “corolla” disk (38mm diameter) with a white pipe cleaner “anther” (Little Crafty Bugs Ltd., UK: product codes CV/CARD-21112 and CV/PC-51619, respectively) protruding 10mm through and perpendicular to the disk (Figure 4). To control for any effect of floral reward on pollinator behaviour, all flowers were made equally rewarding. Approximately 20mg lycopodium spores (mean 20.4 mg ±3.1) (*Lycopodium clavatum* L.: Proteam, Hamburg, Germany; product code Art-Nr 2001) were added to the anthers of all flowers as an unscented pollen surrogate (Table 2,

Appendix 4). Scent treatments were applied by pipetting 60 μ L of floral extract (2 flowers' equivalent) or hexane control onto the artificial corolla and allowing the solvent to evaporate (approx. 5 seconds). Flowers were mounted on the wall of the flight arena opposite the colony entrance using Velcro in a 3 $\times$ 3 Cartesian grid, spaced 100mm apart vertically and horizontally (Figure 3). Preliminary trials revealed that bees preferentially visited flowers at the bottom of an array, so flowers were presented semi-randomly, whereby each treatment was presented on each row in a randomised location (Figure 3). Flowers were replaced after each trial.

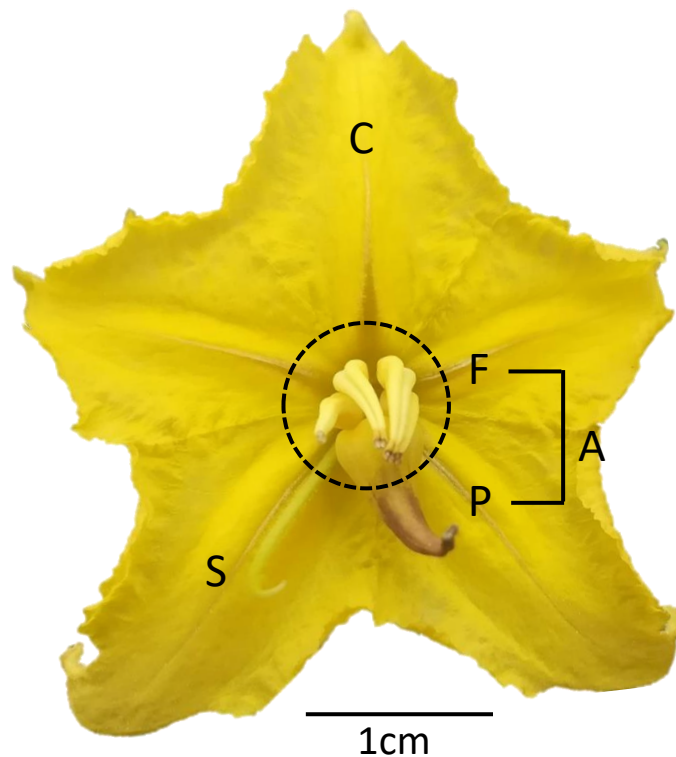


Figure 5. *Solanum rostratum* flower with a large corolla (C), a centrally displaced stigma (S), and distinct heteranthery within the androecium (A) where the four feeding anthers (F) are morphologically distinct from the pollinating anther (P).

General Experimental Protocols

To assess the effect of floral scent on foraging preference and behaviour, bees were allowed to forage freely on an array of flowers varying only in scent. Pollen foraging motivation was increased by withholding pollen from the colony 24 hours prior to a trial. A trial was initiated by releasing one to five workers into the arena. The first bee to deliberately touch a flower became the focal bee and all others were returned to the colony. Floral contact was considered deliberate if bees were under

controlled flight and slowed upon approaching the flower (e.g., not flying chaotically, or out of control having contacted the flight arena). Floral visits were recorded in four categories: 1) touch - bee deliberately contacted any part of a flower with any body part; 2) land - any touch where a bee made contact with 3 or more limbs simultaneously; 3) manipulate - any time a bee landed and exhibited foraging behaviour (i.e., proboscis extension, scrabbling); and 4) buzz - any foraging interaction where a bee sonicated any part of the flower. Behavioural categories form a nested hierarchy, whereby all landings were preceded by a touch, but not all touches resulted in a landing. A floral visit ended once a bee was no longer in contact with the flower with any body part. Trials were terminated when a bee returned to the colony, made 50 floral visits, or did not visit a flower for a period of 5 minutes, whichever came first. Bees were removed from the arena, uniquely numbered, returned to the colony, and allowed a second foraging bout on a subsequent day (Figure 3).

To aid behavioural assessment, audio-visual recordings were started upon first floral interaction with a high-definition digital camera (Panasonic DMC-FZ1000, Osaka, Japan) positioned outside the arena recording at 30 frames per second and connected to an external microphone (H4n Pro Portable, Zoom, Tokyo, Japan) placed in the centre of the arena. During each trial we recorded which flowers bees visited in the array, the behaviour a bee undertook on each flower, floral treatment, and the duration of a visit (a touch was recorded with a duration of 0 seconds as it is an instantaneous interaction).

*Olfactory differences between floral parts of *S. rostratum**

To determine chemical differences between floral organ scents, hexane extracts from the behavioural experiments were analysed by gas chromatography-mass spectrometry (GC-MS). Samples were concentrated from 150 μL to 50 μL in air at ambient temperature (approx. 2 hours). Extracts were analysed with an Agilent GC-MS (7890B GC with 5977A/B MS; Agilent Technologies Inc., CA, USA) using splitless injection (250°C injector temperature) using helium as the carrier gas (1.0603 ml min^{-1} flow rate). The temperature program of the column (Phenomenex 7HG-G015-02-GGA 35 m x 250 μm x 0.1 μm) started at 50°C, was held for 4 minutes, then rose at a rate of 5°C min^{-1} to 230°C, before being held at 230°C for 4 minutes. Compounds were identified by comparing mass spectra and gas chromatographic retention index (Kovats Indices, KI, calculated from the retention times of a chemical standard alkane ladder) with the NIST mass spectral library (NIST, 2014).

Chapter Two. Pollinator Responses to the Scent of Buzz-Pollinated Flowers with Morphologically Distinct Anthers

Spectral differences between floral parts of S. rostratum

To assess whether colour variation in *S. rostratum* pollinating anthers effected their visibility to bees, the reflectance of floral organs was assessed and compared with a bee vision model. Reflectance spectra were produced from the corolla, the tip and base of pollinating anthers, and the feeding anthers (pooled from one flower) of ten individual flowers of *S. rostratum* accessions 10 s 72 / 10 s 74 (Figure 5). Three readings were taken from each organ per flower and averaged. Reflectance spectra were produced using a UV-VIS spectrophotometer (OceanOptics USB4000; Ocean Insight, Orlando, FL, USA) with a pulsed xenon lamp light source (PX-2; OceanOptics) and OceanView v2.0 (OceanOptics) software, and a white standard (Spectralon; NH, United States). One set of three readings, belonging to a pollinating anther base, contained almost all negative readings below 465nm in all 3 replicates (Appendix 1). These were considered as a technical error and excluded from the analyses, leading to only nine observations of pollinating anther base compared to ten observations for all other categories.

Statistical analysis

To determine whether scent treatment influenced bee preference we analysed the proportion of a bees' visits that were made to each scent treatment using a quasi-binomial Generalised Linear Mixed Model (GLMM; glmer function, *lme4* package; Bates *et al.*, 2015) with bee identity fitted as a random effect (Proportion of Visits ~ Treatment + (1 | Bee Identity)). We tested both bees' initial preference and for overall preference, using their first five visits and all visits, respectively. As bees preferred visiting certain scents, we explored whether this preference changed through time with a binomial GLMM. We condensed bees' scent choice to a binomial response variable when analysing both experiments, whereby the androecium was coded "1" and the other treatments coded "0": thereby in Experiment 1 Androecium=1, Corolla=0 and Hexane=0, while in Experiment 2 Pollinating Anther=1, Feeding Anther=1 and Hexane=0. Foraging bout, and floral visit within bout were fitted as fixed effects, and with bee identity as a random effect (Choice ~ Visit + Bout + (1 | Bee Identity)).

The impact of scent treatment on the number of buzzing visits a bee undertook was analysed with a binomial GLMM, using visit behaviour as a binomial response variable (buzz or not), scent treatment as the fixed effect, with bee identity and foraging bout as random effects (Buzzes ~ Scent + (1 | Bee Identity / Bout)). We made pairwise comparisons of GLMM model using *lsmeans* function from *lsmeans* package (Lenth, 2016). To test if bees were buzzing flowers randomly or showing preference to particular treatment(s) for their first buzz, we compared the observed ratio of which treatment received bees' first buzz using a Chi Squared test (chisq.test function, core R *stats* package: R Core Team, 2022). The duration of floral visits was analysed using a GLMM using scent treatment as the

fixed effect, with bee identity and foraging bout as random effects ($\text{Duration} \sim \text{Treatment} + (1 | \text{BeeID} / \text{Bout})$), touches were eliminated from this analysis as they had a duration of zero seconds. As there was a significant effect of scent treatment on floral visit duration, we assessed whether the duration of buzzing visits (mean duration per bee in seconds) varied across scent treatments with ANOVA (`aov` function, *stats* package).

To calculate the capacity of bees to visually discriminate between floral organs, spectrophotometer readings were assessed using a colour space model (Chittka, 1992) using the *pavo* package (Maia *et al.*, 2019). The colour space model compares spectral data against the specific sensitivity of an organisms photoreceptors, at a given illumination (we assumed daylight illumination: Wyszecki & Stiles, 1982) and against a chosen background colour (here green to represent foliage Chittka & Kevan, 2005). The model provides a measure of chromatic contrast ($\Delta S = dS$), i.e., how distinct colours appear to a chosen organisms' vision. Bumblebees are important natural pollinators of *S. rostratum* (Bowers, 1975; Jesson & Barrett, 2005) so we used the trichromatic vision of *Bombus terrestris* (spectral sensitivity is presented in Appendix 3, adapted from Peitsch *et al.*, 1992). Choice experiments have determined that a difference of 0.09 hexagon units is the minimum threshold at which bumblebees can distinguish between colours (Dyer *et al.*, 2008). So, the closer points are when plotted on a colour hexagon the less different they appear to bees and the closer a point lies to the centre the less different it appears from the background colour.

We used R software version 4.2.2 for all analyses (R Core Team, 2022) and figure production. Boxplots (Figure 8 and Figure 11) used the *ggplot2* package (Wickham, 2016), GLMM model prediction plots (Figure 9 and Figure 10) used `plot_model()` function of *sjPlot* (Lüdtke, 2017), and visual space figures (Figure 12, Figure 14 and Figure 15) were produced in *pavo*.

Results

Olfactory Differences Between Floral Parts of S. rostratum

We detected 47 compounds in the floral extracts of *S. rostratum* (Table 2, Figure 6A) consisting of aromatic, cyclic ketone, fatty acid derived, furan, lactone, and terpenoid compounds (Figure 6B). The volume (Figure 6C) and chemical composition of floral VOC differed between floral parts (Figure 6A, Figure 6B, Table 2). The four feeding anthers per flower produced the greatest amount of VOC: 1593.0 ng per flower, equivalent to 398.25 ng per anther (Figure 6C). Two of the 33 compounds in feeding anther extract were unique, 1-Hexadecanol (which contributed 33.08% of VOC) an unknown (KI=1825). Unknown (KI=1864) contributed 33.91% (Figure 6A, Table 2). The sole pollinating anther produced the least VOC: 197.2 ng per flower (Figure 6C, Table 2). This consisted of 35 compounds,

three unidentified compounds were unique (KI= 1144, 1349, 1372) (Figure 6A, Table 2). Ten chemicals were produced by only one anther morph (Table 2). The corolla emitted 443ng of scent containing 28 compounds of which Tetradecanoic acid was most abundant (69.7%). Three corolla-unique compounds: 2-Tridecanone and two unknowns (KI of 1508 and 1830) were detected. γ -Decalactone was found in all floral parts, making 17.3% of feeding anther and 34.0% of pollinating anther extracts, and only 0.5% of corolla extract. As GC-MS analysis used homogenised samples of floral scent from the behavioural assays, samples are pseudo-replicates and not quantitative data which can be directly compared.

Table 2. Mean proportions of compounds detected in extracts of *S. rostratum* floral tissues and Lycopodium spores used in behavioural experiments. Chemical names in bold were verified against standards. Names are italic where Kovats Retention Indexes match those in the NIST webbook.

Compound	Retention time (min)	Kovats index	Lycopodium Spores	Corolla	Pollinating Anther	Feeding Anther	All Anthers
1 <i>Furan, tetrahydro-2,5-dimethyl-</i>	2.877	718	6.7				
2 Unknown	3.003	735	1.0	0.4	1.0	0.1	0.1
3 Unknown	3.536	802		0.4	0.7	0.1	0.2
4 Unknown	4.130	838	0.3	0.6	0.7	0.1	0.1
5 Unknown	4.380	854		0.5	0.1		
6 Z-3-hexen-1-ol	4.411	855		2.2	2.0	0.1	0.5
7 Benzaldehyde	6.648	959	1.5	2.9	1.3	0.3	0.2
8 D-Limonene	8.440	1027	5.8	1.3	2.1	0.2	0.2
9 <i>2-Nonanone</i>	10.382	1094		0.6	3.7	0.5	0.9
10 Linalool	10.570	1101		0.3		0.1	0.4
11 <i>Oxophorone</i>	11.773	1143			2.2	0.6	1.1
12 Unknown	11.826	1144			8.8		
13 Unknown	13.789	1213			1.5	1.6	0.2
14 Unknown	14.224	1229		0.6	0.4	0.6	0.9
15 <i>Nonanoic acid</i>	15.651	1281	8.8				
16 <i>2-Undecanone</i>	16.033	1295		1.1	1.7	0.2	0.4
17 Unknown	16.602	1317			0.2	0.2	0.2
18 Unknown	17.435	1349			0.1		
19 Unknown	18.038	1372			0.5		0.3
20 Unknown	18.572	1393	3.80	0.29	0.79	0.16	0.23
21 Unknown	18.644	1396		2.10			0.04
22 Unknown	19.247	1420					
23 <i>γ-Decalactone</i>	20.432	1469	2.04	0.51	34.03	17.25	24.05
24 <i>2-Tridecanone</i>	21.113	1497		2.55			
25 Unknown	21.372	1508		0.45			

Chapter Two. Pollinator Responses to the Scent of Buzz-Pollinated Flowers with Morphologically Distinct Anthers

26	Unknown	23.442	1597	1.17		0.62	0.37	0.82
27	Unknown	25.034	1670			1.24	0.56	0.90
28	Unknown	25.115	1673	7.24	0.28	0.84	0.08	0.17
29	Unknown	25.562	1694	7.40	0.54	1.79	0.39	0.66
30	Unknown	25.696	1700	1.39		0.58	0.65	1.90
31	<i>trans-Farnesol</i>	26.191	1724			3.97	0.94	0.75
32	<i>Tetradecanoic acid</i>	27.269	1769	12.61	69.67	7.23	1.19	3.06
33	Unknown	27.536	1788	5.96	0.17	1.58	0.24	0.38
34	Unknown	27.710	1796		3.25	0.51		0.22
35	<i>Hexadecanal</i>	28.109	1816	0.92	2.06		0.20	0.12
36	Unknown	28.167	1819			6.36	0.19	0.50
37	Unknown	28.29	1825				1.47	1.98
38	Unknown	28.377	1830		1.08			
39	Unknown	28.522	1837			1.08	0.12	0.33
40	Unknown	28.552	1838		2.99		0.07	0.22
41	Unknown	28.691	1845	1.54		0.13	4.60	4.70
42	Unknown	28.965	1859	21.41	1.02	5.12		
43	Unknown	29.013	1862		0.64	1.42		
44	Unknown	29.066	1864			2.18	33.91	33.73
45	<i>1-Hexadecanol</i>	29.501	1886				33.08	19.54
46	Unknown	29.664	1894	9.07	0.75	1.68	0.14	0.42
47	Unknown	29.970	1910	1.42	0.84	1.96	0.37	0.67

Total VOC Volume (ng) in 30μL of extract	63.8	443.0	197.2	1593.0	1671.4
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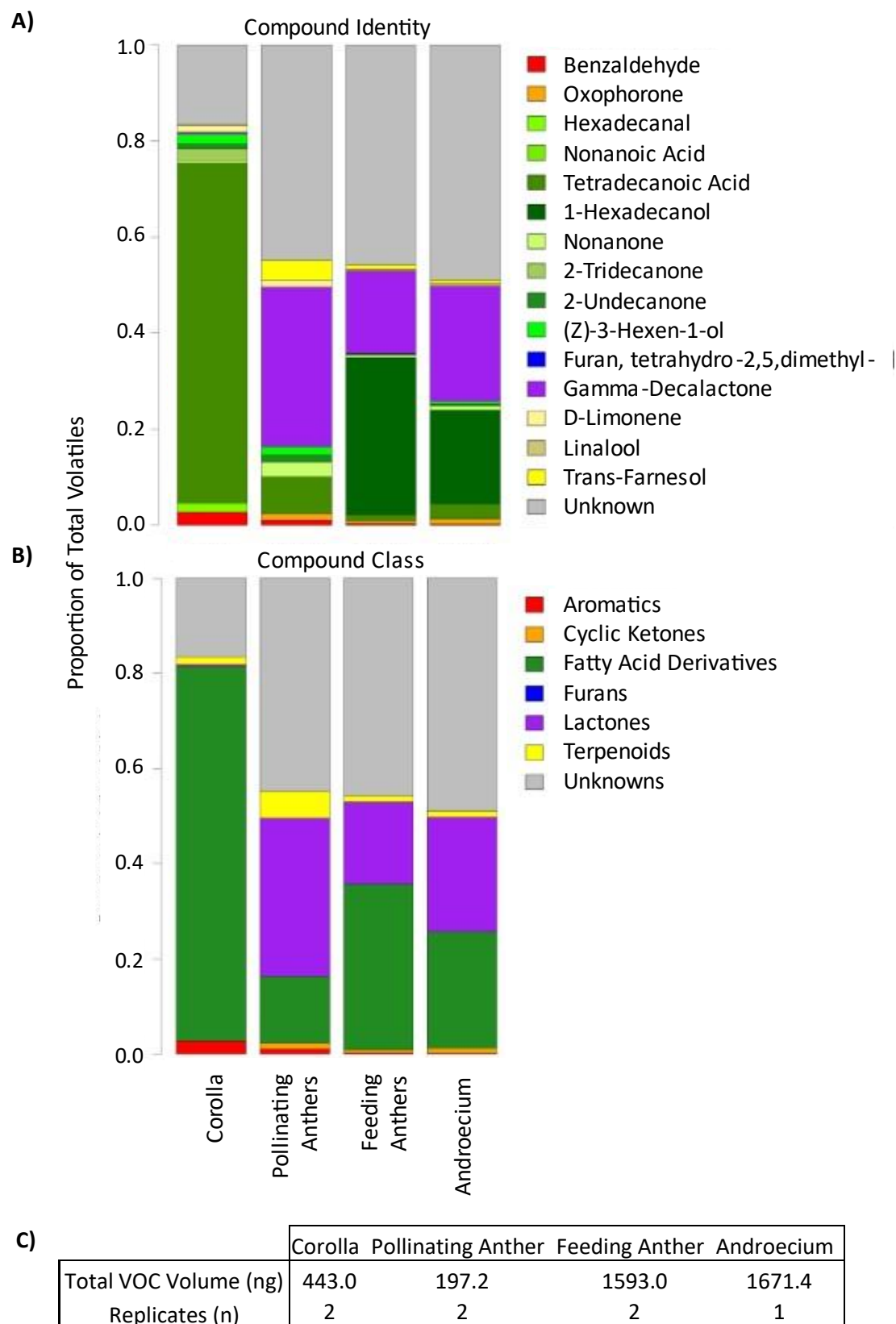


Figure 6. The identity (A) and class (B) of chemical compounds detected in floral scent extracts used for behavioural experiments, and the total VOC volume (C) from one flowers' tissue (30µL of extract).

Experiment 1: Androecium Vs Corolla

In total, 24 bumblebees from two colonies undertook Experiment 1 completing 922 interactions (43 visits per bee ± 21.5 SD; Figure 7) and nine bees completed two foraging bouts. All interactions were initiated by bees deliberately touching a flower, 79% of touches were followed by bees landing, 56% subsequently manipulated flowers and 57% of manipulations resulted in buzzing behaviour (10% of all floral visits; Figure 7).

Experiment 1: Corolla Vs Androecium

Experiment 2: Pollinating Vs Feeding Anther

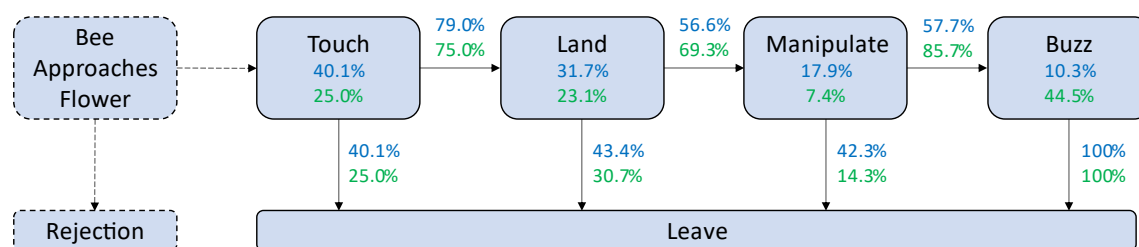


Figure 7. A comparison of the rate at which bees performed each type of behaviour while visiting artificial flowers Experiment 1: Corolla Vs Androecium (blue) and Experiment 2: Pollinating Vs Feeding Anther (green). Floral visits were categorised into a nested hierarchy of bee behaviours: touching, landing, manipulating, and buzzing. The percentage of floral visits which terminated in each behaviour is presented in each box. After performing a given behaviour bees would either progress to perform the next behaviour or depart the flower, the percentage of bees which made each decision is presented beside arrows.

Results show clearly that bees discriminated between scent treatments, preferentially visiting androecium scented flowers (Figure 8A), despite having no initial preference (GLMM: z-value=1.90, $P=0.058$). Bees visited androecium scented flowers approximately 1.4 times more (41.5% of all visits) than corolla or control flowers (29.4% and 29.1% respectively; GLMM: corolla treatment: z-value = -5.15, $P<0.0001$; hexane treatment: z-score= -5.41, $P<0.0001$). Bees' preference for the androecium scent treatment increased between their first and second foraging bout (40.5% and 54.6% of floral visits respectively) (Figure 9A; GLMM: foraging bout; z-value = 3.44, $P<0.0001$), but the visit number in a foraging bout had no significant effect so was dropped from the model.

Scent very strongly influenced whether bees would buzz during a floral visit (Figure 10A). Bees buzzed during 10% of floral visits ($n=94$), with seven bees not buzzing on hexane ($n=5$) or corolla ($n=4$) treated flowers (Figure 7). Androecium scented flowers received a significantly higher proportion buzzing visits (17%) than corolla (6%; GLMM: corolla treatment, z-value = -6.78,

$P < 0.0001$) or control flowers (3.74%; GLMM: hexane treatment: $z\text{-score} = -8.16$, $P < 0.0001$). The observed frequency at which bees performed their first flower buzzes on each treatment (androecium = 15, corolla = 3, control = 2) was significantly different to bees' initial scent preference ($\chi^2_{(2)} = 7.27$, $P < 0.05$).

Bees spent over 4 times longer on androecium treated flowers ($7.2 \text{ seconds} \pm 7.0$) than control or corolla scent (1.6 ± 1.65 and 1.1 ± 1.14 seconds respectively; Figure 11A; GLMM: corolla treatment: $\chi^2 = 7.12$, $P < 0.01$; *lsmeans* pairwise comparisons: anther-corolla $p < 0.01$; anther-control $P < 0.05$, corolla-control $P > 0.05$).

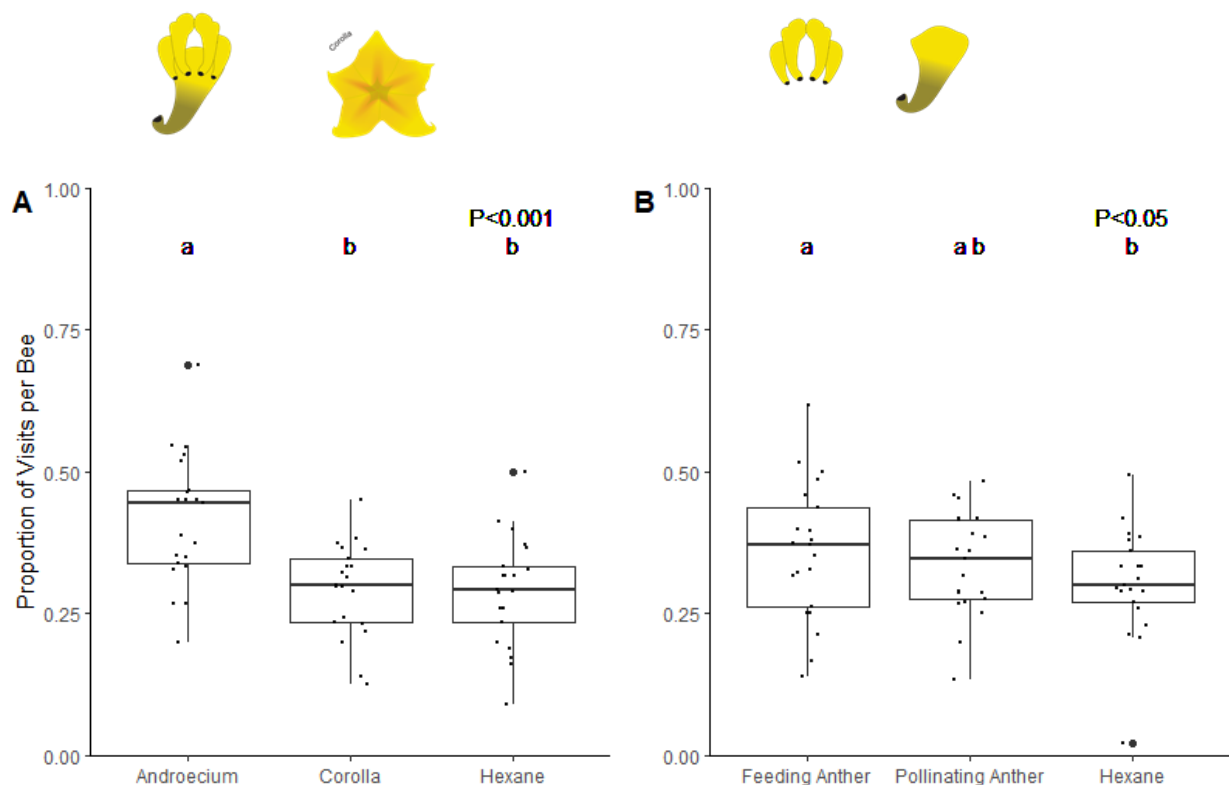


Figure 8. Bees preferentially visited androecium scented flowers to corolla and control scents in Experiment 1: Androecium Vs Corolla; **(A)**. Between the organs of the androecium, Experiment 2: Feeding vs Pollinating Anther **(B)**, bees preferred scents of feeding anthers over the control, but pollinating anthers were not significantly different from feeding anther or the control. Hexane was the control in both experiments, letters indicate significant differences between treatments with P-values indicated on figures.

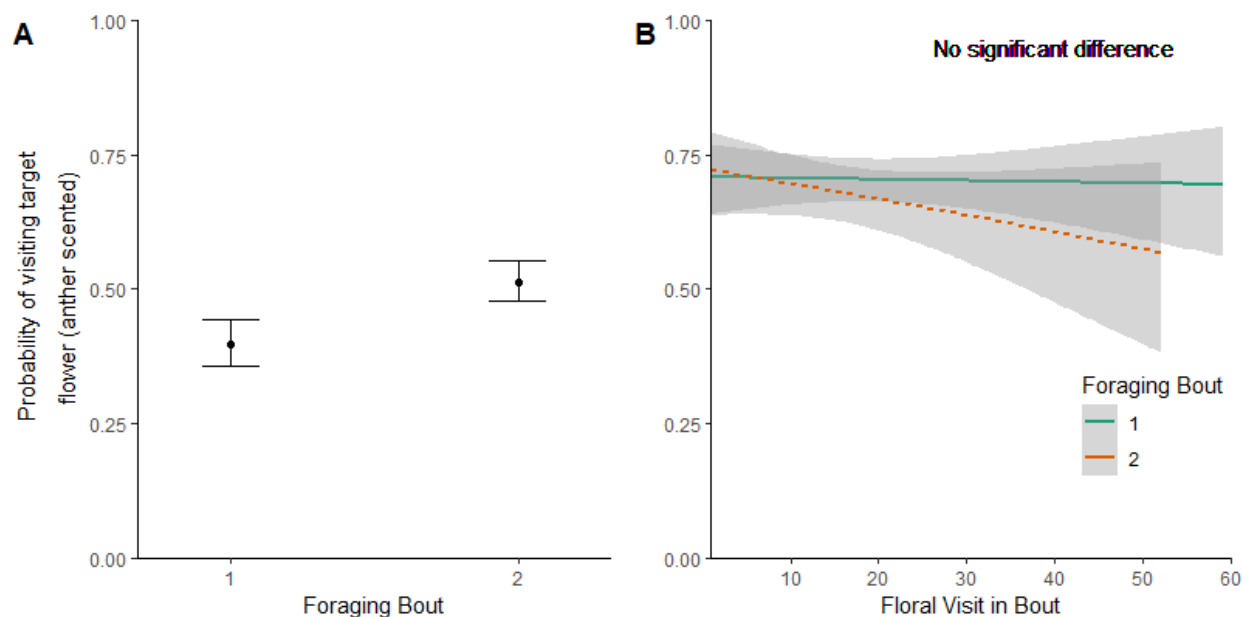


Figure 9. The preference of *Bombus terrestris* foragers for androecium scent was higher in foraging bout 1 than bout 2 of Experiment 1: Androecium Vs Corolla (**A**), as preference did not change with visit in a bout this was not presented here. No change in preference was detected between bouts or with visit in Experiment 2: Feeding vs Pollinating Anther (**B**). Data are GLMM model predictions with error bars showing standard errors. Letters indicate significant differences, P-values are indicated on figures. See Figure 3 for clarification in the difference between floral visit and foraging bout.

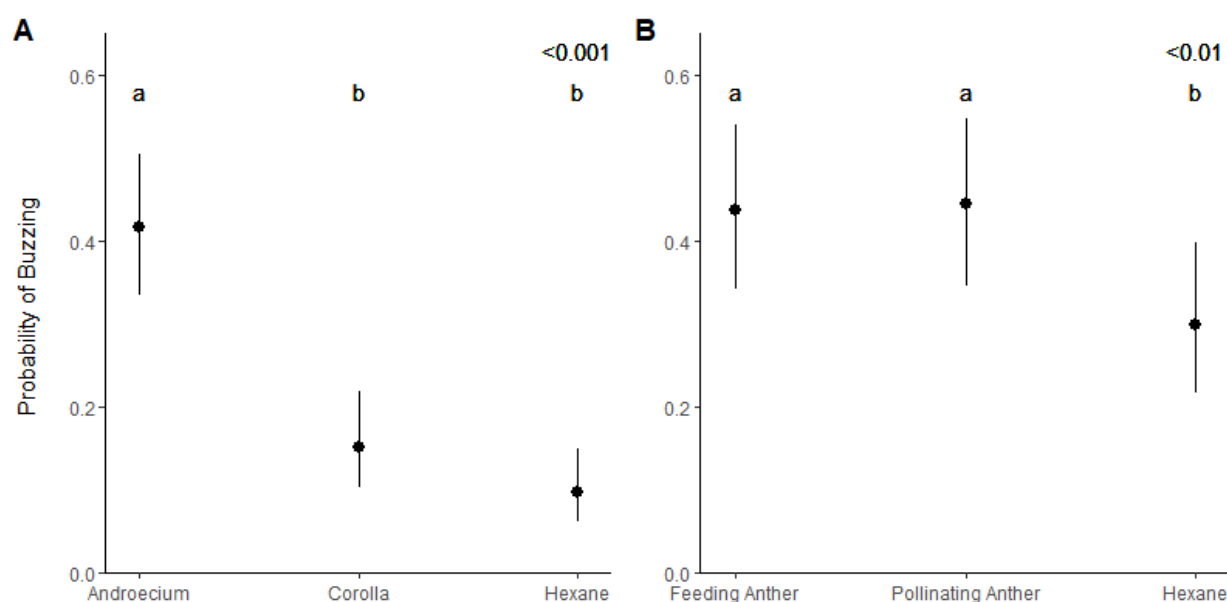


Figure 10. The probability of a bee buzzing when visiting flowers treated with androecium scent extract was significantly higher than other treatments in both Experiment 1: Corolla vs Androecium (**A**) and

Experiment 2: Feeding vs Pollinating Anther (**B**). Dot and whisker plots were produced with GLMM model predictions, letters denote significant differences with P-values indicated on figures. The control was hexane.

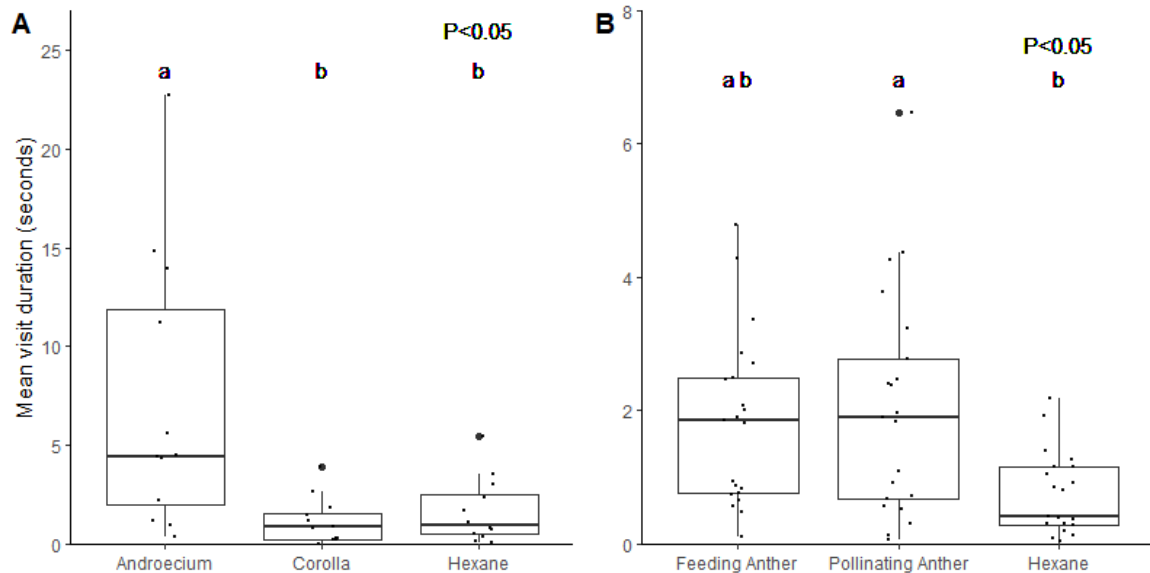


Figure 11. *Bombus terrestris* foragers spent longer visiting artificial flowers treated with androecium scent in Experiment 1 (Androecium Vs Corolla) (**A**), while in Experiment 2: Feeding vs Pollinating Anther (**B**) visits to pollinating anthers were significantly longer than the control but feeding anther treatment was not significantly different from either other treatment. Letters indicate significant differences, P-values indicated on figures. Hexane was the control treatment.

Experiment 2: Feeding vs Pollinating Anther

Experiment 2 was undertaken by 25 bumblebees from 2 colonies. In total 885 floral visits were completed (42 visits per bee ± 27.7 SD) with ten bees completed two foraging bouts. 75% of touches were followed by bees landing, 69% subsequently manipulated flowers and 86% of manipulations resulted in buzzing flowers (44% of all floral visits; Figure 7).

Scent treatment had a small but significant influence on bees' flower choice (Figure 8B) bees preferring feeding anther scented flowers (35.9% of visits) than control flowers (30.5%; GLMM: hexane treatment: z-value = -2.30, $P < 0.05$). Pollinating anthers received an intermediate proportion of visits (33.6%) which was not significantly different from other treatments ($P > 0.05$). Bees did not demonstrate an initial preference (GLMM: z-value = -0.94, $P = 0.35$) and there was no significant

change in preference through time ($P>0.05$), either within a single bout or between bouts (Figure 9B).

Bees were almost twice as likely to buzz flowers treated with extracts from both anther type (Figure 10B; feeding anther $15.9\% \pm 5.1$, pollinating anther $14.4\% \pm 4.2$ respectively) than the hexane control ($8.5\% \pm 4.2$; pairwise comparisons of GLMM: control-feeding anther: $z\text{-value} = 3.27$, $P<0.01$; control-pollinating anther: $z\text{-score} = 3.30$, $P<0.01$). The two anther treatments were equally likely to be buzzed ($P>0.05$). We recorded no difference between the observed frequency at which bees performed their first flower buzzes (feeding anther = 4, pollinating anther = 9, hexane = 6) and bees' initial scent preference (chi-squared $P>0.05$).

Bees spent longer visiting pollinating anther scented flowers (2.0 seconds ± 1.7) than control flowers (0.8 seconds ± 0.6 ; Figure 11B; GLMM: $X^2 = 7.2$, $P<0.05$; *lsmeans* pairwise comparisons: $P<0.05$).

There was no difference between the duration of feeding anther visits (1.8 seconds ± 1.7) and control visits ($P=0.08$), or between the anther treatments.

Spectral Differences Between Floral Organs

Solanum rostratum flowers had yellow, UV-reflecting corollas (Figure 5, Figure 12, Figure 13). The androecium, however, is UV absorbing (low reflectance below 450 nm), with yellow feeding anthers and pollinating anther base. In contrast, the pollinating anther's tip is brown, with much lower reflectance than other floral parts. All organs demonstrated high reflectance between 500 – 700 nm and maximum reflectance at 700 nm (Figure 12) which would appear red to humans but is not visible to bees (Appendix 3).

Bees can theoretically distinguish between all floral parts using colour: separation in the colour space model (Figure 14) corresponding to perceptual differences over the 0.09 hexagonal units threshold (Figure 15, Appendix 2). Bees should be most able to distinguish pollinating anthers tips from all other floral parts (Figure 15, Appendix 2) due to large perceptual differences. Feeding anthers, corollas and pollinating anther bases are more perceptually similar (Figure 15, Appendix 2: corolla-feeding anther = 0.36, corolla-pollinating anther base = 0.40, feeding anther-pollinating anther base = 0.59).

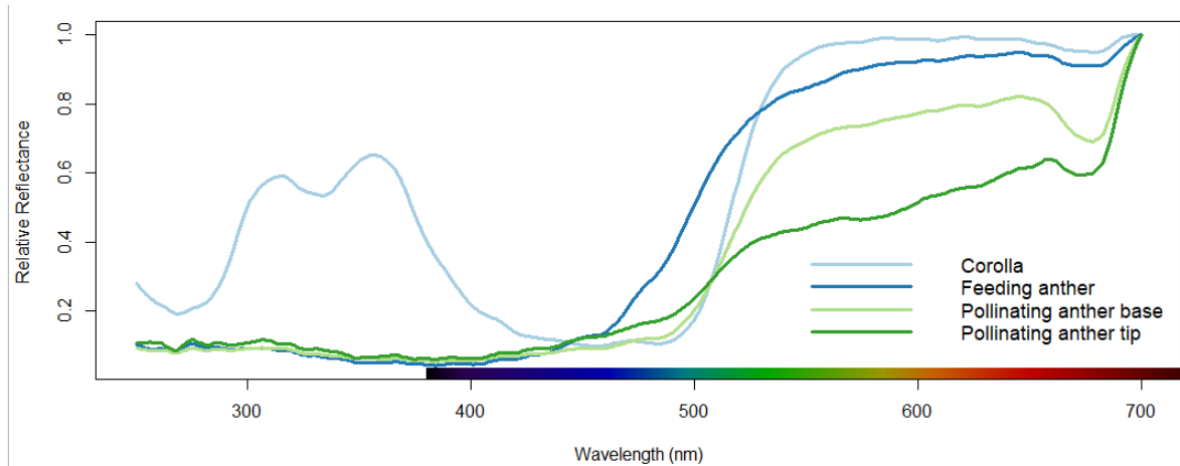


Figure 12. Mean reflectance spectra of *Solanum rostratum* differed between all floral organs (n=10 per organ except pollinating anther base where n=9). Maximum reflectance of all organs was at 700nm which bees cannot see (see Appendix 3), corollas are unique with high reflectance in the ultraviolet (300-375nm). Line colours are arbitrary, colour spectrum at the base presents trichromatic human colour vision.



Figure 13. *Solanum rostratum* corollas reflect ultraviolet light while anthers are UV absorbent. Photograph credit: Mario Vallejo-Marin.

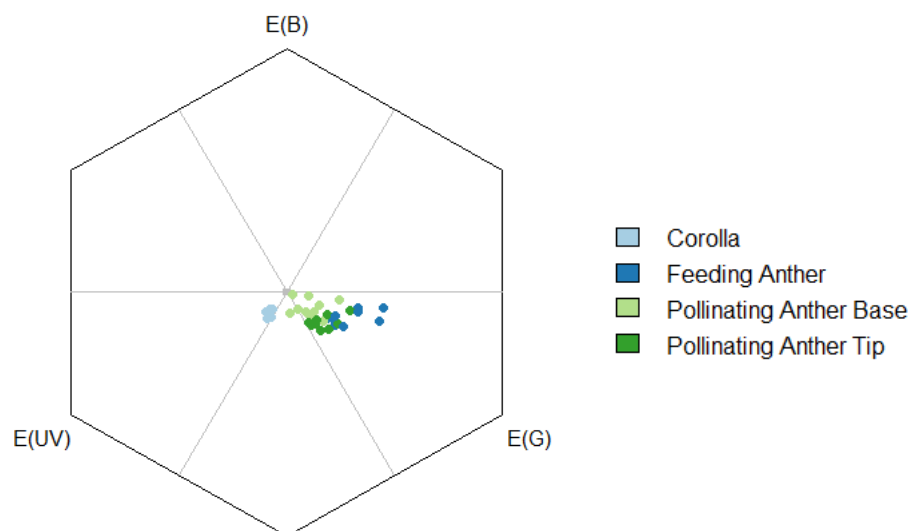


Figure 14. *Solanum rostratum* floral organ reflectance plotted to bee vision (*Bombus terrestris*) colour space. The axes of the colour hexagon represent the relative excitation of the three colour photoreceptor cells (E) of *B. terrestris* blue (B), green (G) and ultraviolet (UV). The further a point is from the centre the more visible it is against the background (green) and the further apart two points are the more distinguishable they are in bee vision. Point colours are arbitrary.

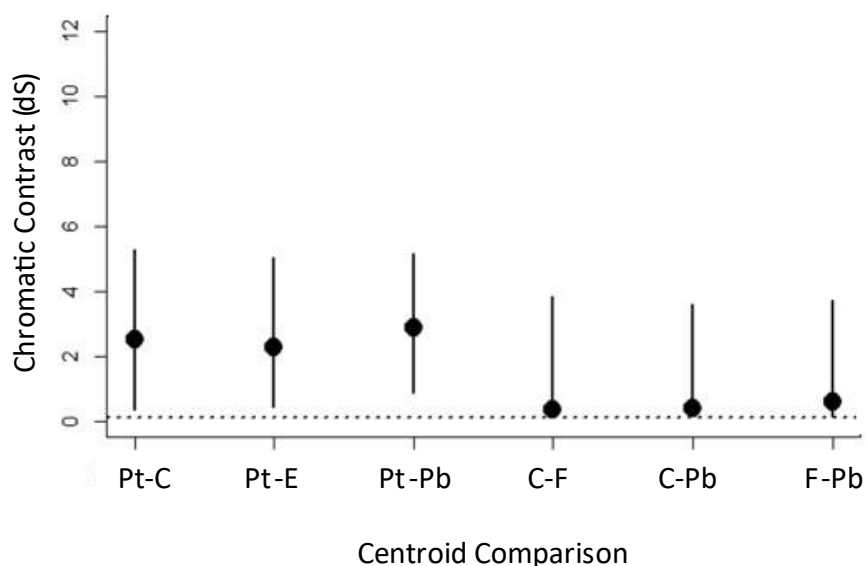


Figure 15. *Bombus terrestris* can, theoretically, visually distinguish between all *Solanum rostratum* floral organs as chromatic contrast is greater than the perceptual threshold of 0.09 (dashed line, defined by Dyer et al., 2008). Points present mean chromatic contrast and whiskers the maximum and minimum difference for each organ pair. Letters denote floral organs: Pb= pollinating anther base, Pt= pollinating anther tip, C= corolla, F= feeding anther.

Discussion

Heteranthery and plant-pollinator signalling

Flowers produce complex signals to communicate with pollinators (Raguso, 2004a), with distinct floral organs and tissues often contributing different components to these attractive displays (Russell *et al.*, 2018). In heterantherous flowers, anther specialization appears to incorporate morphology (e.g., shape and size) (Vallejo-Marín *et al.*, 2009), colour (e.g., saturation and hue), and scent (Solís-Montero *et al.*, 2018; Basso-Alves *et al.*, 2022), which are hypothesised to increase efficiency of pollen transfer. We tested pollinator response to scent extracted from different floral organs in the heterantherous *Solanum rostratum* revealing significant behavioural differences. The androecium was most attractive to pollinators and stimulated longer visits compared to the corolla. Surprisingly, within the androecium, feeding and pollinating anthers were both equally attractive to bees and stimulated pollen collecting behaviour (i.e., buzzing), despite the feeding anthers producing eight times greater VOC volume than pollinating anthers. The composition of VOC also varied between anther morphs, both in terms of presence and of chemical abundance. This appears to contradict the division of labour hypothesis of heteranthery which suggests divergent specialisation of different anthers to the two different roles of pollination: rewarding pollinators or reproduction (Vallejo-Marín *et al.*, 2009). Indeed, pollinating anther treated flowers contained fewer organs ($n=2$) than feeding anther ($n=8$), suggesting pollinating anthers individually produce more scent, perhaps on account of their greater size. However, in *S. rostratum*, scent producing osmophores are located at the base of all anthers in the flowers' centre (Solís-Montero *et al.*, 2018). The proximity of anthers and the diffusion of scent may mean differentiation of scent cues between anther morphs cannot be detected by pollinators and so prevents divergent selection.

Although we found no difference in the response of bees to scent produced by the feeding and pollinating anthers in *S. rostratum*, visual cues varied significantly between organs, with feeding anthers more visually conspicuous to bees compared to pollinating anthers based on spectral analysis. The tips of pollinating anthers, the most important part to pollen export, are the least visible floral part to bees which indicates visual specialisation between anther morphs. Taken altogether, our results indicate that floral organs of heterantherous plants may share functions in pollinator attraction and positioning: some signals differentiate the feeding and pollinating anthers (e.g., vision, scent volume), while others are shared (e.g., scent regulated attraction and behaviour). Qualitative analyses provide an important initial exploration of the use of scent by heteromorphic flowers (Basso-Alves *et al.*, 2022, this study). However, as bees will make foraging decisions based on the relative abundance of chemicals in blends of the same composition (Lawson *et al.*, 2018) further

quantitative studies (e.g., Solís-Montero *et al.*, 2018) will be critical to a complete understanding of scent use by heterantherous species.

Signal coordination and buzz pollination

The combination of scent and visual signals attract pollinators and encourages them to land and forage in the centre of the flower thus optimizing pollen export to conspecifics. Corollas emitted little scent but were large and visually attractive with significant ultraviolet reflectance, probably playing a role in long distance attraction as in *Solanum houstonii* (Russell *et al.*, 2018). The contrast between the peripheral UV-reflecting corolla and the central UV-absorbing anthers fits with the general bull's eye pattern found in some bee pollinated flowers (Tunes *et al.*, 2021; Lunau *et al.*, 2021; Basso-Alves *et al.*, 2022). The UV absorbent, yellow centre may represent pollen mimicry (Lunau, 2000) as reported in buzz-pollinated flowers (Basso-Alves *et al.*, 2022). Attractive signals of the androecium encourage bees to land in the centre of the flower where scent stimulates buzz pollination. The resulting vibrations expel pollen from feeding anthers where it gathers on the ventral abdomen of bees and is easily collected and so effectively lost to the plants (Parker *et al.*, 2015). The close grouping of all anthers in the flower allows transmission of vibrations to pollinating anthers (Nevard *et al.*, 2021) which avoid pollinator attention by being centrally displaced and visually cryptic and thus may not be buzzed directly. Pollinating anther structure and position allows pollen placement in so called "safe sites": areas of the bees' body which are difficult to groom but are accessible to conspecific stigmas (i.e., the dorsal abdomen, Figure 2) (Vallejo-Marín *et al.*, 2014; Koch *et al.*, 2017; Solís-Montero & Vallejo-Marín, 2017). Floral signalling and structure may also influence florivores, further impacting reproductive success (Vega-Polanco *et al.*, 2020). This is one of the major findings of our study, and further highlights how different organs of heterantherous flowers interact in a relatively complex manner.

Bees learn to distinguish flowers which contain equal rewards but require specialist behaviour for efficient collection

The ability of bees to learn is well documented (Whitney *et al.*, 2009b; Clarke *et al.*, 2017; Harrap *et al.*, 2020, 2021) so it should be unsurprising that bees appeared to learn a scent preference, from no initial preference, in both of our experiments. However, in previous studies learning occurred when flowers were unequal in some reward associated characteristic (i.e., reward quantity (Ruedenauer *et al.*, 2015), quality (Nicholls & Hempel de Ibarra, 2017), reliability (Austin *et al.*, 2019), palatability (Muth *et al.*, 2016a), mechanical defences (Lunau *et al.*, 2015), nutrition (Vaudo *et al.*, 2016), and inclusion of chemical components (Couvillon *et al.*, 2015) and toxins (Jacquemart *et al.*, 2019)), whereas the artificial flowers of this study contained an equal reward amount. So why did bees

develop a preference? Exploration of novel flower types despite sensory biases or prior learning is a strategy bees use to discover new resources (Evans & Raine, 2014) so we expected all flower types to be visited, and bees were observed gathering rewards from all flowers as they were designed such that reward was exposed and collectable by general foraging behaviour (scrabbling: Russell *et al.*, 2017). However, as floral buzzes proved much more effective at collecting pollen, treatments which elicit more buzzes (i.e., all androecium treatments) will be perceived as more rewarding by bees. This may explain bees' increased preference for the androecium compared to the corolla treatment but not when choosing between different anther treatments. In Experiment 1 bees had a $\frac{1}{3}$ chance of selecting high rewarding flowers (i.e., androecium Vs corolla and control treatments), while in Experiment 2 this was $\frac{2}{3}$ (i.e., feeding anther and pollinating anther Vs control). When the chance of making an incorrect choice is greater, so is the pressure to visit the "correct" flower (i.e., one where scent stimulates buzzing). This is a key finding which must be considered when designing future pollen foraging and buzz-pollination experiments.

Change in bee preference between foraging bouts suggests that, at least some of bee learning occurred in the colony as in Molet *et al.* (2009). Learning may occur as pollen is unloaded or via feedback mechanisms with individuals within the colony, as in other social hymenopterans (Cassill & Tschinkel, 1999; Behmer, 2009; Ruedenauer *et al.*, 2015). This may be why there is ongoing debate as to whether bees can detect pollen or assess its quality while foraging (reviewed in Nicholls & Hempel de Ibarra, 2017). Many experiments have not allowed bees a memory consolidation period (e.g. Ruedenauer *et al.*, 2015; Muth *et al.*, 2016; Russell *et al.*, 2016). Others pair pollen stimuli with sucrose rewards (e.g. Grüter *et al.*, 2008; Arenas & Farina, 2012; Ruedenauer *et al.*, 2015), an unnatural situation which provides immediate positive feedback which may stimulate learning (Cook *et al.*, 2005). Hence the debate surrounding whether bees can assess the quality and quantity of pollen while foraging remains unresolved. Further exploration of the divergent rules for foraging on alternative rewards (i.e., pollen or nectar) may provide interesting insight into memory formation and learning in bumblebees (e.g., Konzmann and Lunau (2014), Nicholls & Hempel de Ibarra (2017)), especially if different types of foraging behaviour, flower complexity and reward immediacy were incorporated.

Social learning occurs through the observation and imitation of other individuals (Akers, 1973) and is well documented in bees (Worden & Papaj, 2005; Goulson *et al.*, 2013; Alem *et al.*, 2016) providing a non-exclusive alternative explanation for learning outside experimental arrays. For example, scents introduced to the nest by returning foragers are learned by flower naive bees and preferred in future foraging excursions (Molet *et al.*, 2009; Farina *et al.*, 2022). Although rewards used in this experiment were unscented, scent from the array may still enter the nest influencing the choices of

future foragers. We found no change in bee preference depending on the order in which they undertook the experiment, but scent introduced to the colonies by returning foragers may have reinforced learning between a bees' first and second foraging bout.

Foraging bees commonly explore all flower types in a patch or array (Stout *et al.*, 1998; Austin *et al.*, 2019), presumably as an adaptation to uncertainty and change in natural systems. Seemingly contradictory to the hypothesis that scent triggers buzzing, we observed bees buzzing unscented flowers (as reported by Russell *et al.*, 2018), albeit much less than androecium scented flowers. We propose three non-mutually exclusive hypotheses for why bees buzzed control flowers: the Novel Flower Hypothesis, the Generalized Behaviour Hypothesis, and the Scent Seepage Hypothesis.

The Novel Flower Hypothesis suggests that bees try new behaviours (e.g., buzzing) when encountering new floral types based on the adaptive nature of bee foraging (Dafni *et al.*, 2010; Russell *et al.*, 2017). This hypothesis predicts first buzzing to occur on each flower type at the rate they appear in the array (here $\frac{1}{3}$). Conversely, the Generalized Behaviour Hypothesis suggests initially bee buzzing requires stimulation (e.g., androecium scent) but once learned can be applied to all flowers encountered independent of the trigger. This explanation predicts bees first buzz should be made on androecium scented flowers. We found that bees were more likely to perform their first buzz on androecium treated flowers than by chance. This allows us to eliminate the Novel Flower Hypothesis quite confidently while supporting the Generalized Behaviour Hypothesis. The Scent Seepage Hypothesis proposes that cues which trigger behaviour (i.e., androecium scent triggers buzzing) are detectable at locations distant to the source (e.g., via diffusion) where they stimulate buzzing. The random arrangement of treatments in the array prevents analysis of the Scent Seepage Hypothesis, but it could be tested by varying the distance between flowers in an array or with a Y-maze. Regardless, this result confirms a key finding of Russell *et al.* (2018): that scent plays a key role in stimulating buzzing in buzz-pollinated flowers. It would be interesting to explore whether scent can trigger buzzing of non-poricidal flowers (Russell *et al.*, 2017).

In summary, our research provides evidence that the signals emitted from different floral organs operate together to improve reproductive fitness through the manipulation of pollinator attraction, orientation, and behaviour. We suggest that such fine control of pollinators may be especially important in plant species with highly specialised flower morphologies. Examples of such specialised morphologies include flowers in which pollinator positioning is key to effective pollen import and/or export (e.g., heterantherous species) or in which unusual pollinator behaviour is required for effective pollination (e.g., poricidally dehiscent anthers requiring buzzing for pollen removal). We also demonstrate that the different floral organs of heterantherous flowers have distinct functions in

attracting pollinators via different signals (e.g., shape, colour, scent, reward), and that each organ has multiple functions. Signals from a single organ or tissue are specialised to transmit divergent information to a receiver by using different communication channels (i.e., pollinating anthers producing attractive scents while being visually cryptic) may be a more general rule of communication between two organisms. This divergent specialisation is unlikely to be unique to plant-pollinator interactions.

Acknowledgements

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Chapter Three. Pollen removal changes floral scent of buzz-pollinated *Solanum* flowers

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Abstract

Floral scent influences the recruitment, learning, and behaviour of floral visitors. Variation in floral scent can provide cues of the amount of reward available or whether a flower has been visited recently and may be particularly important in species with visually concealed rewards. In many buzz-pollinated flowers, tubular anthers opening via small apical pores (poricidal anthers) visually conceal pollen and appear similar regardless of pollen quantity within the anther. We investigated whether pollen removal changes scent composition and emission rate in seven taxa of buzz-pollinated *Solanum* (Solanaceae). We found that pollen removal reduced overall scent emission. Moreover, in *S. lumholtzianum*, pollen removal reduced the emission of specific compounds (linalool and farnesol). Our findings suggest that scent could be used as a cue by visitors of buzz-pollinated flowers as it contains information on potential pollen availability.

Keywords

Chemical ecology, concealed rewards, headspace, honest signal, linalool, plant-pollinator signalling, volatile organic compound, pollination.

Introduction

Scent is a key floral trait that can influence plant reproductive success by attracting pollinators or manipulating behaviour and learning (Wright & Schiestl, 2009; Schiestl, 2010a; Russell *et al.*, 2018). Floral scents comprise a mixture of volatile organic compounds (VOC), of which >2,000 have been identified, and may have broad or specific activity (Knudsen *et al.*, 2006; El-Sayed, 2019; Gfrerer *et al.*, 2021). Pollinators can detect the presence and determine the relative abundance of VOC, allowing them to discriminate between complex scent blends when making foraging decisions (Byers *et al.*, 2014; Zhao *et al.*, 2022). Therefore, the diversity and abundance of VOC allows flowers to attract specific pollinators, and elicit specialized behaviours such as copulation in sexual mimicry (Schiestl *et al.*, 2003), or vibrations in buzz pollination (Russell *et al.*, 2018).

Recent advances in floral chemical ecology demonstrate that scent is a dynamic trait that varies across space and time, allowing plants to modulate where and when signals are emitted (Burdon *et al.*, 2015, 2020; García *et al.*, 2021). Spatio-temporal analyses of floral VOC have revealed functions beyond pollination such as defence against predators (Scala *et al.*, 2013), parasites (Morinaga *et al.*, 2009) and pathogens (Huang *et al.*, 2012). Temporal variation in scent includes daily cycles found in many pollination systems (Raguso & Willis, 2005; Morinaga *et al.*, 2009; Balao *et al.*, 2011), where peak emission of VOC coincides with key pollinator foraging activity (Chapurlat *et al.*, 2018). Longer term changes include community scale effects caused by drought (Jaworski *et al.*, 2022) or where plants flowering early in the season, when pollinators are rarer, produce stronger scents than those flowering when pollinators are in abundance (Filella *et al.*, 2013). Permanent scent changes occur in response to herbivory (Kessler *et al.*, 2011) and florivory (Vega-Polanco *et al.*, 2020) which impacts reproductive success, as well as post-pollination to protect seeds from predation (Schiestl *et al.*, 1997; Theis & Raguso, 2005; Burdon *et al.*, 2015). Experimental studies have also shown that floral scent attracts pollinators across spatial scales: airborne scent plumes act at long range (Dobson, 1994), scent guides on petals orient bees at medium range (Dötterl & Jürgens, 2005), and scented rewards act at close range (e.g., nectar or pollen; Burdon *et al.* 2020; Dobson *et al.* 1999).

Variation in the scent composition may occur based on reward availability, and may lead to compound concentrations rising or falling as rewards are depleted and replenished (Dobson, 1988; Raguso, 2004a; Pacini & Hesse, 2005; Wright & Schiestl, 2009; Burdon *et al.*, 2015, 2020). We would expect pollinators to prefer scents associated with rewards because this facilitates more efficient foraging by avoiding depleted flowers. In fact, olfactory and visual signals associated with rewards were likely present in the most primitive flower-pollinator interactions based on fossil evidence (Crepet, 1983). That flowers typically produce signals from non-rewarding tissues suggests that

scented reward production is either not beneficial or unfeasible (Raguso, 2004a,b, 2008a; Leonard *et al.*, 2011b). This may be due to relatively small quantities of reward being unable to emit sufficiently large signals to attract pollinators or that adding secondary metabolites to rewards may render them distasteful or toxic (Stevenson *et al.*, 2017; Rivest & Forrest, 2019; Jacquemart *et al.*, 2019).

Alternatively, rewards may be contained within floral structures for protection (e.g., from environmental damage or detrimental floral visitors), which restrict or prevent signal transmission in one or more senses (Buchmann *et al.*, 1983) e.g., nectar of bilabiate flowers (Westerkamp & Claßen-Bockhoff, 2007), pollen in poricidal flowers (Buchmann *et al.*, 1983), or both in keel flowers (Westerkamp, 1997).

Poricidal flowers occur in approximately 22,000 species across at least 65 families, including some crops (e.g., tomato and kiwi) (Buchmann *et al.*, 1983; De Luca & Vallejo-Marín, 2013). In contrast with typically longitudinally dehiscent flowers, poricidal flowers release pollen through pores or slits in response to vigorous vibrations: buzz-pollination (Buchmann *et al.*, 1983). This adaptation effectively restricts pollen access to approximately 74 bee genera, containing ca. 58% of bee species, reported to be capable of buzz pollination (Hedtke *et al.*, 2013; Cardinal *et al.*, 2018). The enclosing structures of poricidal flowers conceal pollen and always appear full (Buchmann *et al.*, 1983), yet bees visit rewarding flowers preferentially (Buchmann & Cane, 1989) where scent stimulates buzzing (Russell *et al.*, 2017). As plants can change their scent quickly (Farré-Armengol *et al.*, 2014) and bees may use this trait to discriminate between rewarding and unrewarding flowers (Clarke *et al.*, 2013, 2017; Harrap *et al.*, 2017, 2021), avoiding inconsistent or unrewarding flowers (Austin *et al.*, 2019), here, we hypothesise that poricidal plants' scent varies with pollen availability.

Using *Solanum*, a large genus of nectarless flowers that conceal their pollen inside poricidally dehiscent anthers, we asked whether floral scent changes depending upon reward availability. Scent would be expected to fluctuate with the presence of reward in the buzz-pollinated flowers of *Solanum* to provide a cue of reward availability which bees could use when foraging. Results from headspace analyses of floral scent of seven closely related *Solanum* taxa reveal specific changes in the emission and composition of VOC associated with pollen presence and suggest that scent acts as a signal of reward availability in plants with concealed floral rewards.

Methods

Study System

Solanum is a large genus (c. 1,350 species) used as a model system for investigating buzz pollination (Frodin, 2004; Särkinen *et al.*, 2013; Russell *et al.*, 2016b; Vallejo-Marín, 2019). *Solanum* flowers are nectarless, often hermaphroditic (Harris, 1905; Knapp, 2002), and release pollen via small distal anther pores in response to vibrating insects (King & Buchmann, 1996). Here we studied *Solanum* section *Androceras*, a small clade of self-compatible, annual or perennial herbs native to Mexico and the USA (Whalen, 1978a; Stern *et al.*, 2010). We selected seven taxa for analyses of scent composition, which have been previously used in detailed analyses of floral variation and mating system (Whalen, 1978a, 1979; Vallejo-Marín *et al.*, 2014; Kemp & Vallejo-Marín, 2021), and the response of floral scent to florivory (Vega-Polanco *et al.*, 2020). *Solanum rostratum* (Dunal) and *S. citrullifolium* (A. Braun) produce large floral displays consisting of many flowers open simultaneously, with strong heteranthery present in each flower where one anther is clearly larger than the others (Figure 16) (Stern *et al.*, 2010; Vallejo-Marín *et al.*, 2014). *Solanum fructu-tecto* (Cav.) and *S. heterodoxum* (Dunal) produce small displays consisting of few small flowers open simultaneously across the plant, rarely more than one flower open per inflorescence at any time with shorter anthers and less pronounced heteranthery (Figure 16). The display size of *S. grayi* is dependent on the presence of the large displaying *S. lumholtzianum* (Bartlett): in allopatric regions *S. grayi* produces large displays (*S. grayi* subspecies *grandiflorum*) but in sympatric locations *S. grayi* produces small displays only (*S. grayi* ssp. *grayi*).

Plant Material

Experimental plants were grown at the University of Stirling. Seeds were collected from wild populations in Mexico between 2007-2010, except *S. citrullifolium* which was obtained from Radboud University's Experimental Garden and GeneBank Solanaceae collection, Nijmegen, The Netherlands (Table 3) (Vallejo-Marín *et al.*, 2014; Nunes *et al.*, 2021). Germination was stimulated by soaking seeds for 24 hours at room temperature in 1,000 ppm aqueous gibberellic acid solution (GA3; Sigma-Aldrich, Dorset, UK). Seeds were then drained and planted individually in seed trays (2×2×5cm) containing Modular Seed Growing Medium Compost (William Sinclair Horticulture PLC, Lincoln, UK) and placed in a growth chamber with 16-24°C temperature cycle and 75% relative humidity. Plants were illuminated at 4.8-9.5klx in an 18:6 hour Light:Dark cycle. After 2-4 weeks seedlings were transferred to individual 1.5 litre pots (15cm diameter) containing a 4:1 mix of All Purpose Growing Medium and Perlite Standard (William Sinclair Horticulture PLC, Lincoln, UK) in a pollinator proof glasshouse. Plants were fertilised weekly with Tomorite Concentrated Tomato Food

(Levington, Surrey, UK), watered as needed 3 times a week, maintained between 16-25°C and illuminated for ≥ 16 hours per day by sunlight supplemented with compact-fluorescent lights.

Floral Headspace Sampling

Floral scent samples were collected between May and September 2019. Floral headspace (i.e., airborne scent naturally emitted by the flower) was continuously sampled for 6 hours starting between 06:00-08:00, covering the period of peak pollinator visitation to wild plants (Solís-Montero *et al.*, 2015) which we hypothesised to coincide with peak attractant VOC emissions (following Muhlemann *et al.*, 2014). For each sample collected, material from a single plant was enclosed within an odourless 3.2 litre PET Bag (Multi-Purpose Cooking Bag Sainsbury's Supermarkets Ltd., London, UK: see Stewart-Jones & Poppy, 2006). A mains-operated vacuum-pump (FB65540, Fisher Scientific) pulled air through a charcoal filter into the bag to remove contaminants and extracted air through a collection filter at a rate of $2,000 \text{ cm}^3 \text{ min}^{-1}$. Filters consisted of a 4mm i.d. Pasteur pipette containing Porapak Q (200 mg, 50–80 mesh; Waters, Milford, MA 01757, USA)) held between plugs of silanized glass wool. Control filters were treated and handled identically to experimental ones except that no air was drawn through them. All filters were wrapped in aluminium foil and transported to the Natural Resources Institute, Chatham Maritime, UK.

Pollen Scent Sampling

We hypothesised that pollen was a source of floral VOC, so pollen of *S. citrullifolium*, *S. lumholtzianum* and *S. rostratum* was mechanically removed by vibrating anthers within a 2ml Eppendorf tube using an electric toothbrush (Braun Oral-B Type 3756; Oral-B, Redwood City, CA, USA) operating at approximately 1,000 Hz for 30 seconds or until pollen expulsion ceased, whichever took longer. Samples were pooled by species in 2ml Eppendorf tubes and stored at -18°C until 0.5ml was collected. Pollen was then mixed with 1ml hexane, left for 1 hour to dissolve VOC then centrifuged for 1 minute at 5,000 RPM. The supernatant was pipetted into clean 2.5 ml glass vials and transferred to the Natural Resources Institute for analysis. No VOC were detected in the solvent extracts from pollen of *S. citrullifolium*, *S. lumholtzianum* and *S. rostratum*.

Floral Headspace Sampling Experimental Conditions

Headspace samples were collected from unmanipulated virgin flowers, and flowers from which pollen was removed. Pollen was removed from individual flowers as above, no more than 20 minutes before headspace sampling started. Care was taken to ensure stigmas were excluded from the Eppendorf to avoid pollination which can cause floral VOC changes (Theis & Raguso, 2005). Both conditions were simultaneously sampled from separate individuals of the same accession. Four

samples were collected per treatment per species, except *S. lumholtzianum*, which was only sampled three times per treatment due to difficulty getting sufficient plants to flower. Individuals were used only once.

Table 3. Seed source locations for *Solanum* section *Androceras* studied.

Taxa	Accession	Location	Latitude (N)	Longitude (E)	Elevation (m)	Herbarium Specimen
<i>S. citrullifolium</i>	199-7-1 199-7-7 199-7-9	Nijmegen, Solanaceae Collection	NA	NA	NA	NIJ: 894750197
<i>S. heterodoxum</i>	11-PTM-14 11-PTM-15	Teotihuacan Archaeological Site, Mexico State	19.685 19.685	-98.843 -98.843	2,284 2,284	RBGE: E01009043; E01009044; E01009045
<i>S. rostratum</i>	10-s-77 10-s-79 10-s-81	San Miguel, Queretaro State	20.901	-100.705	2,033	RBGE: E01009047; E01009048
<i>S. fructu-tecto</i>	10-AH-9 11-CU-4	Atitalaquia, Hidalgo State Ciudad University, Mexico City	20.066 19.394	-99.216 -99.192	2,090 2,311	ECOTAP: HET1905
<i>S. grayi</i> ssp. <i>grayi</i>	07-s-19-4b 07-s-19-5b 07-s-19-6b	Guamúchil, Sinaloa State	25.340	-107.951	93	IBUNAM: MEXU1229 199
<i>S. grayi</i> ssp. <i>grandiflorum</i>	08-s-78 08-s-79	Tejupilco, Mexico State	18.852	100.131	1,375	RBGE: E01009041; E01009042
<i>S. lumholtzianum</i>	07-s-57 07-s-59	Guamúchil, Sinaloa State	25.340	-107.951	93	NA

Volatile Analysis

Collection filters were extracted in 1ml of dichloromethane. Decylacetate (5µg) was added to each sample as an internal calibration standard. VOC samples were analysed with flame ionization detection (FID) using an Agilent 7890 GC (Agilent Technologies Inc., Santa Clara, CA, USA), with a split/splitless inlet using helium as the carrier gas (2.4ml min⁻¹ flow rate). The column (Agilent DBWax 30m x 0.32mm x 250µm) was held at 60°C for 2 mins before ramping to 240°C by 6°C min⁻¹. Data was captured and analysed with EZChrom Elite (Agilent). Results were calibrated against pure synthetic standards of the compounds. Peak identities were further confirmed by analysing samples

by gas chromatography/mass spectrometry, using a Varian GC (Varian Medical Systems, Palo Alto, CA, USA) coupled to a Varian 2000 ion trap and a DBWax column (Agilent, 30m x 0.25mm x 250µm).

Analysis

To determine variation in total floral VOC emission rate between plant taxa and in response to pollen presence, we analysed the per flower total VOC emissions (ng min⁻¹) with two-way ANOVA (*aov* function, stats package) using emission as the dependent variable with plant taxa and pollen presence as the explanatory variables (emission ~ taxa × pollen presence). Type III corrections were applied (*Anova* function, car package) to account for an unbalanced design, as one fewer VOC collection was made for *S. lumholtzianum* in each treatment than all other species. Between-species comparisons were explored with Tukey's honest significant difference test (Tukey's HSD: *TukeyHSD* function, stats package). The effect of pollen presence within species was analysed using student t-tests as these are logical, *a priori* pairs. To assess whether floral odour differed between species and if taxon-specific scents changed based on pollen presence, we used a 2-way MANOVA (*manova* function, stats package), analysing emission of the 5 detected VOC per flower (ng minute⁻¹) as dependant variables with plant taxa and pollen presence as explanatory variable. We further investigated the MANOVA results with 2-way ANOVA of with Bonferroni corrections (*summary.aov* function, stats package, Alpha=0.008). R version 4.2.1 (R Core Team, 2022) was used for all analyses and figures generated in R ggplot2 package (Wickham, 2016).

Linalool has two naturally occurring stereoisomers with different biological properties (Raguso & Pichersky, 1999) while farnesol has four geometric isomers (Yu *et al.*, 2005). Unfortunately, due to methodological limitations we were unable to distinguish the isomers in this study. (Z)-3-hexenol, methyl phenylacetate and geraniol were identified in control and experimental filters and were hence considered contaminants and excluded from all analyses (Appendix 5). Butylated hydroxytoluene was also considered a contaminant and excluded from analysis as it is not a known natural product from plants (DF personal observation), though produced by algae (Babu & Wu, 2008), it is an antioxidant found in PVC tubing as used in this study (Hill *et al.*, 2003).

Results

Total Emission of Floral Scent

The total amount of floral VOC emitted by the flowers varied by two orders of magnitude between taxa, from 18.5-767.9 ng minute⁻¹, with *S. lumholtzianum* emitting 3-42 times more VOC than other taxa ($F_{6,40}=17.1$, $P<0.001$; Tukey's HSD $P<0.001$) (

Table 4, Figure 16, Appendix 7). Pollen containing flowers emitted 5-1,039% (*S. grayi* ssp. *grayi* and *S. lumholtzianum*, respectively) more scent than those without pollen ($F_{1,40}=7.7$, $P<0.01$) (Figure 16).

The between-treatment difference was significant only in a single species, *S. lumholtzianum* (interaction: $F_{1,40}=9.5$, $P<0.001$); t-test: $t_{(6)} = 7.9$, $P<0.05$) (Appendix 8), and not between or within any other taxa (Figure 16).

Composition of Floral Scent

Five VOC detected in the floral headspace in *Solanum* section *Androceras* varied significantly between taxa (MANOVA: $F=4.03$, $P<0.001$) (Figure 17, Figure 18, Table 3). Two of these, linalool and farnesol, also reduced in response to pollen removal ($P<0.001$), with significant differences in the response to pollen removal detected between taxa (interaction $P<0.001$) (Figure 18; Appendix 5, Appendix 7; Appendix 9). Linalool was only detected in the headspace of pollen containing flowers of the three large-displaying taxa, *S. lumholtzianum*, *S. grayi* ssp. *grandiflorum* and *S. citrullifolium*, where it comprised 64.9%, 2.3% and 1.5% of the with-pollen floral scent, respectively (

Table 4). *Solanum lumholtzianum* emitted significantly more linalool than other taxa ($F_{6,40}=45.28$, $P<0.001$) (Figure 17A), where linalool emissions responded dramatically to pollen removal decreasing from 498.1 ng min⁻¹ to 0 ng min⁻¹ ($F=35.45$, $P<0.001$), and was responsible for the significant interaction ($F=45.28$, $P<0.001$; Tukey's Pairwise HSD: $P<0.001$). Linalool was also detected only in pollen containing flowers of *S. grayi* ssp. *grandiflorum* and *S. citrullifolium* (i.e., linalool was not detected in flowers without pollen), but emissions were low and the change was not statistically significant (

Table 4). Farnesol was detected in all taxa and varied significantly with pollen presence ($F_{6,40}= 7.182$, $P<0.05$) (Figure 16, Figure 17B,

Table 4), with the greatest difference observed in *S. lumholtzianum* where pollen containing flowers emitted 36 times more farnesol than those without (171ng minute⁻¹ and 5ng minute⁻¹ respectively; Tukey's Pairwise HSD, $P<0.05$) (Figure 17, Figure 18). There were no other significant differences between or within species, however the interaction was significant ($F_{6,40}= 2.9202$, $P<0.05$).

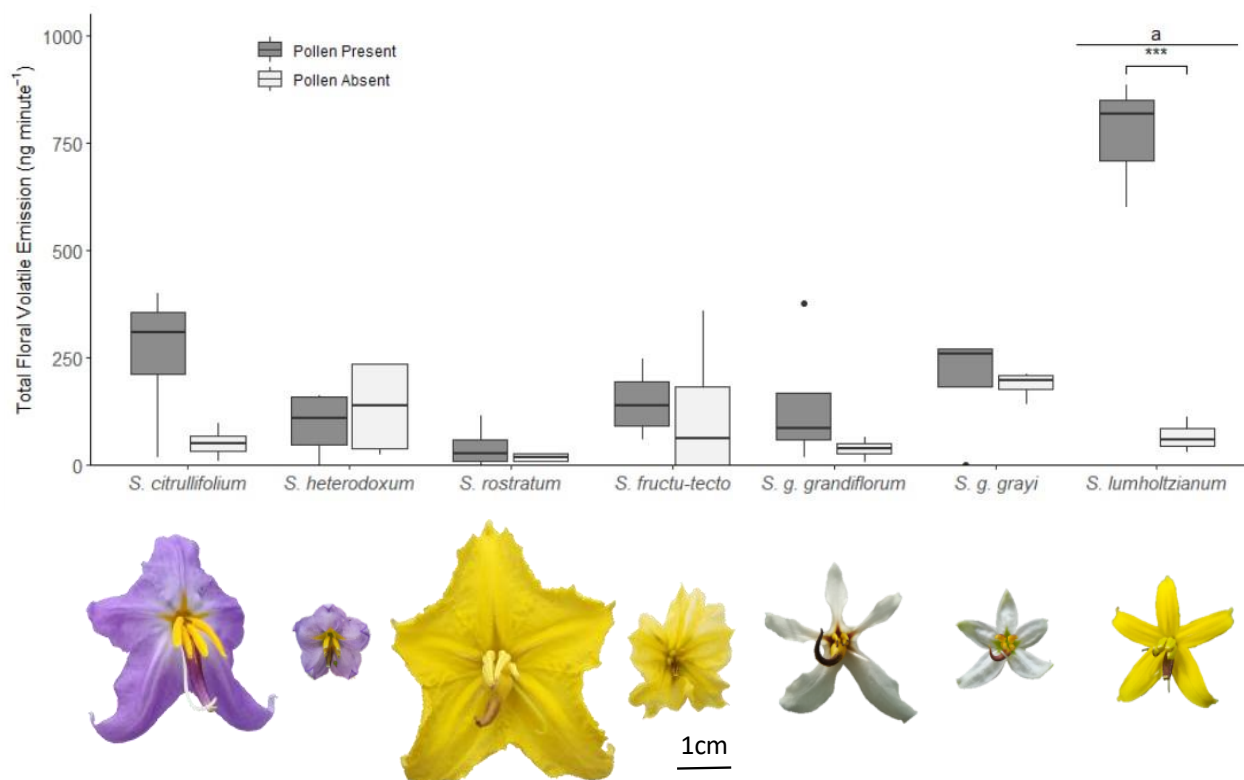


Figure 16. Floral VOC emission rate of *Solanum* section *Androceras* with and without pollen.

Solanum lumholtzianum emitted significantly more VOC than all other taxa and pollen containing flowers of this species emitted significantly more VOC than those without pollen. Asterisks signify within-species significant differences (* $P < 0.05$, *** $P < 0.001$) and letters denote between-species differences ($P < 0.05$). Sample size=4 per treatment per species, except *S. lumholtzianum* where $n=3$.

Three further VOC showed significant differences between species but not associated with pollen presence. Hexenyl acetate was detected in all taxa except *S. fructu-tecto* and varied significantly between taxa ($F_{6,40}=4.463$, $P < 0.005$) (Figure 16, Figure 17D,

Table 4). Hexenyl acetate accounted for 11.4% of the scent in *S. grayi* ssp. *grayi* ($44.0 \text{ ng}^{-1} \text{ min}^{-1}$), significantly more than all other taxa (Tukey's Pairwise HSD, $P < 0.05$) except *S. citrullifolium* (mean $23.3 \text{ ng}^{-1} \text{ min}^{-1}$). Farnesal was detected in all taxa with significant differences in emission between species: *S. fructu-tecto* produced $99.1 \text{ ng}^{-1} \text{ min}^{-1}$, 7.8 times more than *S. rostratum* ($F_{6,40}= 2.409$, $P < 0.05$; Tukey's Pairwise HSD, $P < 0.05$) (Figure 17E,

Table 4). Caryophyllene was recorded in all taxa except *S. rostratum* and *S. lumholtzianum* and varied significantly between taxa ($F_{6,40}= 2.390$, $P < 0.05$) (Figure 17F,

Table 4) although no pairwise comparisons were significant (Tukey's Pairwise HSD $P > 0.05$).

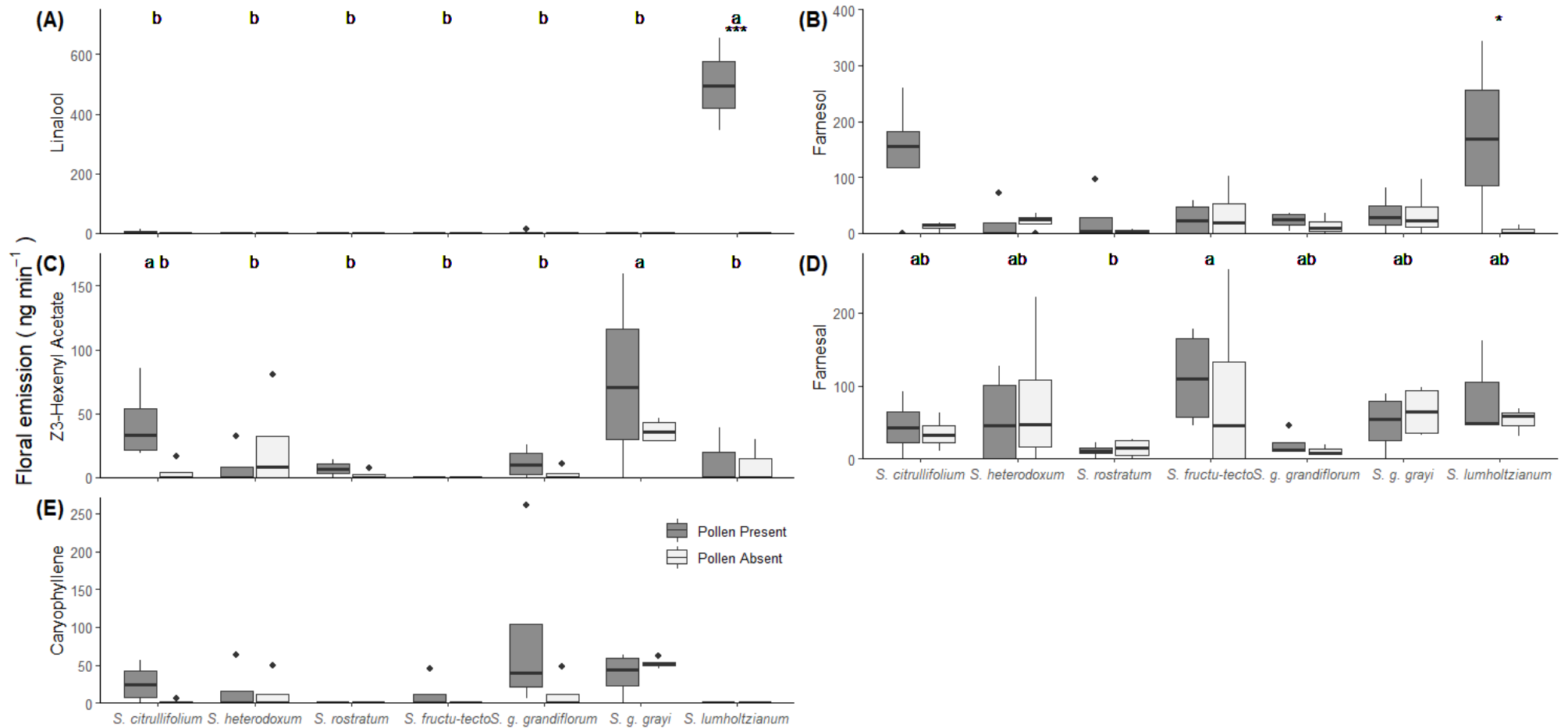


Figure 17. Emission rate of five VOC emitted by *Solanum* section *Androceras* flowers with and without pollen. Linalool (A) was detected only in pollen containing flowers of *S. citrullifolium*, *S. rostratum* and *S. lumholtzianum*. *Solanum lumholtzianum* emitted more linalool than all other taxa which decreased significantly after removal of pollen. Farnesol emission (B) in *S. lumholtzianum* was also significantly higher in pollen containing flowers. Significant between-species differences were detected in the floral emission of hexenyl acetate and farnesal (C and D respectively) but not in caryophyllene. Asterisks denote within-species statistically significant differences (* = $P < 0.05$, *** = $P < 0.001$) and letters denote between species differences ($P < 0.05$).

Table 4. Total floral volatile organic chemical emission per flower of taxa in *Solanum* section *Androceras* with and without pollen, and mean emission of individual chemical compounds (ng minute⁻¹ flower⁻¹). VOC were identified by comparison with chemical standards and by comparing Kovats Indices against online databases (Appendix 6). Standard deviation presented in brackets (±).

	<i>S. citrullifolium</i>		<i>S. heterodoxum</i>		<i>S. rostratum</i>		<i>S. fructu-tecto</i>		<i>S. g. grandiflorum</i>		<i>S. g. grayi</i>		<i>S. lumholtzianum</i>	
Sample size (n)	4		4		4		4		4		4		3	
Condition	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen
Total Emission (ng minute ⁻¹ flower ⁻¹)	259.3 (145.6)	51.9 (31.2)	96.4 (68.5)	135.4 (100.8)	43.1 (44.4)	18.5 (9.2)	147.3 (71.2)	121.5 (147.1)	142.5 (138.3)	38.1 (22.1)	197.4 (114.5)	188.1 (28.2)	767.9 (120.9)	67.4 (33.4)
Z3-Hexenyl	42.4	4.1	7.9	24.0	6.6	1.7	-	-	11.0	2.7	75.2	36.5	12.9	9.8
Acetate	(26.6)	(7.0)	(13.8)	(33.0)	(5.1)	(3.0)			(10.5)	(4.6)	(60.9)	(7.7)	(18.3)	(13.8)
Linalool	4.0 (4.9)	-	-	-	-	-	-	-	3.3 (5.7)	-	-	-	498.1 (126.8)	-
Caryophyllene	26.2 (22.5)	1.4 (2.4)	15.9 (27.5)	12.5 (21.6)	-	-	11.3 (19.5)	-	86.4 (102.4)	11.9 (20.7)	38.1 (25.1)	52.3 (6.3)	-	-
Farnesal	44.0 (33.7)	34.8 (19.4)	54.8 (56.2)	78.7 (86.4)	11.1 (8.2)	14.4 (11.6)	110.8 (58.1)	87.4 (105.8)	20.0 (14.7)	10.4 (6.0)	49.7 (35.0)	64.3 (30.2)	85.6 (54.2)	52.9 (15.7)
Farnesol	142.7 (92.6)	11.7 (7.1)	17.7 (30.7)	20.3 (12.6)	25.3 (41.4)	2.3 (2.5)	25.3 (25.9)	34.2 (41.2)	21.8 (13.0)	13.2 (13.5)	34.3 (29.6)	35.1 (37.3)	171.2 (140.5)	4.7 (6.7)

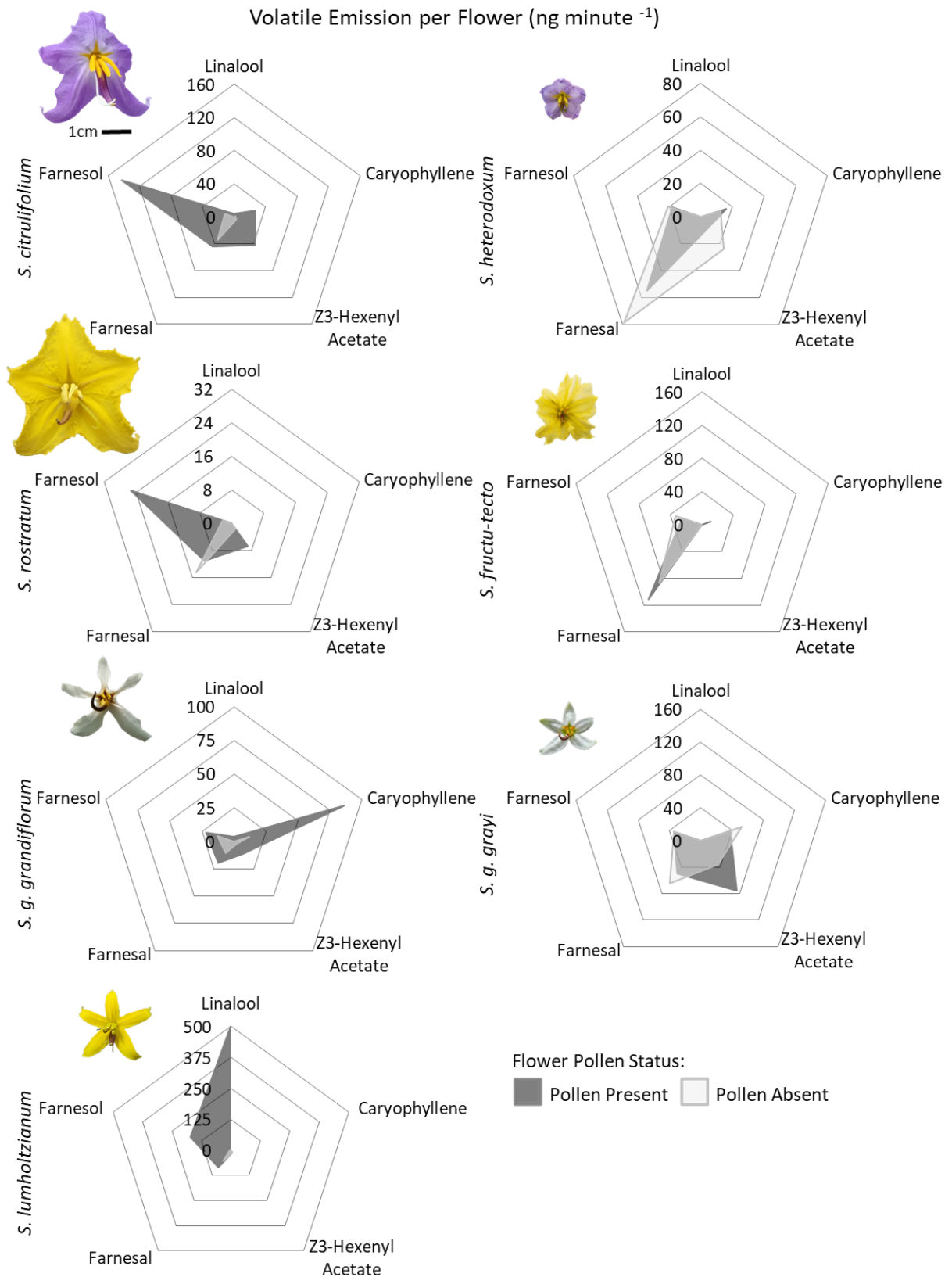


Figure 18. Floral scent composition differed between the taxa of *Solanum* section *Androceras* and changed after pollen was removed. Linalool was emitted by pollen containing flowers of *S. lumholtzianum*, *S. grayi* ssp. *grandiflorum* and *S. citrullifolium* but was not detected in the headspace of any flowers without pollen. Linalool and farnesol emission in *S. lumholtzianum* reduced significantly upon pollen removal.

Discussion

We set out to address whether floral scents vary depending upon the availability of visually concealed pollen, which could be used by foraging pollinators. Specifically, we asked whether seven poricidal taxa change their floral scent upon pollen removal. Species of *Solanum* section *Androceras* have long been known to be chemically distinct due to the evolution of novel flavonoid biosynthetic pathways during the diversification of the group (Whalen, 1978a). It is therefore unsurprising that between-species differences were found in three chemicals detected in this study: linalool, hexenyl acetate and farnesol. Of far greater consequence was our finding that vibrational pollen removal causes significant changes in *Solanum lumholtzianum* VOC, with reductions in the emission of total floral scent and of individual chemicals, namely linalool and farnesol. Indeed, linalool was only detected in the headspace of flowers containing pollen, present also in *S. citrullifolium* and *S. grayi* ssp. *grandiflorum*, but absent in flowers without pollen. Other authors have described correlations between floral traits and nectar (Knauer & Schiestl, 2015; Gervasi & Schiestl, 2017; Parachnowitsch *et al.*, 2019) but this is the first demonstration in pollen rewarding plants.

Traits that correlate with reward quality or quantity may be considered honest signals as they provide signals which foragers can rely upon to indicate the location of the most rewarding food sources. In our study we demonstrated that emissions of VOC correlated with the presence of pollen in at least one species, *S. lumholtzianum*. Indeed, the presence of linalool in the scent of pollen-containing flowers of two further species suggests that there may be a conserved mechanism for honest signalling in the group, but further investigation is needed to resolve this. Honest reward signals are expected to evolve and persist where they are beneficial, by increasing plant export and receipt of pollen, and they will be used and learnt by pollinators where they improve the efficiency of pollinator learning, handling, and profitability (Dobson *et al.*, 2000; Raguso, 2004b). Where these conditions are not met or are rendered irrelevant by plant transitions away from scent-oriented pollinators or zoophily entirely, as has been suggested in some species of section *Androceras* (Vallejo-Marín *et al.*, 2014), scent signalling would be expected to break down (Parachnowitsch *et al.*, 2012; Gervasi & Schiestl, 2017). Alternatively, if the cost of signalling is greater than the benefit it provides these signals will break down, such as through increased herbivory or florivory. This can occur either in the lifetime of the plant due to developmental plasticity (Schiestl *et al.*, 2014) or across generations due to local adaptation (Theis & Adler, 2012). Therefore, we would not expect all species of pollen-only flowers to present honest scent signals, rather those which rely most heavily on scent-oriented pollinators like bees.

We offer two hypotheses as potential mechanisms for the observed scent changes: 1) Scented Pollen Hypothesis, and 2) Damage Hypothesis. The Scented Pollen Hypothesis states that pollen itself is a source of some floral VOC, the removal of which changes overall flower scent. Although pollen VOC were not detected in this study it is a common trait in angiosperms where it contributes to overall floral scent (Ruedenauer *et al.*, 2015). However, the few studies that have measured the contribution of pollen to overall floral scent in poricidal flowers have been equivocal. In some species of *Solanum*, no VOC were detected in pollen (this study; Solís-Montero *et al.*, 2018), while some VOC were detected in other species (Kessler & Halitschke, 2009; Palmer-Young *et al.*, 2019). These differences may be due to pollen scent being a highly variable, species variation, or due to methodological differences. It may also be that pollen of poricidal species is unscented. Pollen scent is emitted by a pollen grain coating (i.e., pollenkitt) in most angiosperms (Pacini and Hesse, 2005), but pollenkitt is greatly reduced or absent in flowers that are buzz- and wind-pollinated (Buchmann *et al.*, 1983; Timmerman & Barrett, 2021). Hence, pollen in buzz-pollinated flowers such as in *Solanum* could be unscented. Furthermore, it may be that the structure of poricidal flowers, with small anther pore apertures and internal chambers (Buchmann & Cane, 1989; Burkart *et al.*, 2014; Russell *et al.*, 2016a), reduces potential benefits of scent production in pollen. Previous research showed that *S. rostratum* scent is produced by osmophores on the exterior of anthers, the composition which did not vary with pollen presence. Future work across multiple poricidal flower families using a combination of standardised collection methods (e.g., solvent extraction, solid-phase microextraction and headspace collection) would help resolve whether the VOC associated with pollen are derived from the pollen itself or other organs of the androecium or flower (D'Arcy *et al.*, 1990; Sazima *et al.*, 1993; Passarelli & Bruzzone, 2004; Solís-Montero *et al.*, 2018; Falcão & Stehmann, 2018).

The Damage Hypothesis offers an alternative explanation of why scent changed in response to pollen removal, postulating that damage to anthers due to pollen extraction by insect vibrations or experimental extraction leads to scent changes. Plants detect and respond to environmental stimuli (Trewavas, 2014; Atamian *et al.*, 2016; Allievi *et al.*, 2021) and to damage (i.e., herbivory: Kessler *et al.*, 2011; Schiestl *et al.*, 2014) by producing secondary metabolites which alter plant physiology or signal to conspecifics and natural allies. Physiological responses to herbivory can result in reduced pollinator attraction through emission of repellent compounds or reduced emission of attractive ones (Strauss *et al.*, 1999; Adler *et al.*, 2006; Kessler & Halitschke, 2009; Kessler *et al.*, 2011; Schiestl *et al.*, 2014). Florivory causes scent change in *S. rostratum*, reducing pollinator and herbivore attraction but not reproductive success (Vega-Polanco *et al.*, 2020). Further investigation would help disentangle which mechanism causes VOC changes, for example by comparing scent of flowers from

which pollen has been extracted by buzzing bees, by damaging and non-damaging experimental methods (i.e., sound waves Kemp & Vallejo-Marín, 2021 or indirect vibrations Nunes et al., 2021) and by vibrating anthers without extracting pollen accompanied by a comprehensive investigation of whether pollen emits VOC directly. However, regardless of the mechanism which causes scent change, bees will use perceptible differences between flowers to inform foraging decisions (Kunze & Gumbert, 2001; Rains *et al.*, 2008; Clarke *et al.*, 2013; Pahl *et al.*, 2013; Harrap *et al.*, 2020). This might, therefore, represent an important but overlooked mode of plant-pollinator signalling in poricidal flowers worthy of further investigation, especially in relation to pollinator foraging behaviour.

Our study suggests that scents associated with reward (i.e., pollen) could represent an example of honest signalling used by flowers with concealed rewards and in pollen-only flowers. We showed that headspace of the pollen-only, poricidal flowers of *S. lumholtzianum* change in response to pollen removal although more work is required to show whether this is attributed to the loss of pollen emitted VOC. Emission of both linalool, a chemical with well characterised pollinator attractive function, and farnesol positively correlated with pollen availability, and could be used by pollinators when making foraging decisions.

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Chapter Four. Use of scent by pollen foraging bees

Abstract

Pollen is the male gametophyte of all flowering plants and in over 10% of species it is the only reward for pollinators. A rich source of complex nutrients (e.g., proteins and lipids), pollen is consumed directly or fed to young. However, thus far research has focussed on pollinators foraging for nectar (a solution of simple nutrients e.g., sugars and amino acids) and little is known about pollen foraging. Scent is a key component of floral signals which informs pollinators' foraging decisions and can trigger specialist pollen collecting behaviour in bees (i.e., buzz pollination), but the role of scent in pollen foraging is not well understood. Here we explore how pollinators use floral scent when foraging for pollen. We tested the role of scent in decision making by pollen foraging bumblebees (*Bombus terrestris*) by offering them a pollen surrogate in an array of artificial flowers with and without scent. We tested whether scent influenced bees' choice of artificial pollen-only flowers, and whether flower constancy over an extended period of foraging was influenced by whether or not reward was paired with scent. Our results showed that bees initially use scent to inform selection of novel flowers, but that they learn to overcome this preference if scented flowers prove unrewarding. Furthermore, we observed that pollen collection by buzzing was stimulated by the presence of either reward or scent. These findings develop our understanding of this key ecological interaction and the methods developed herein could facilitate future pollen foraging research.

Keywords

Pollen, behavioural ecology, bee foraging, choice, *Bombus terrestris*, chemical ecology, foraging decisions, learning, behavioural ecology.

Introduction

Almost 90% of flowering plants depend on pollinators for reproduction to some extent (Ollerton *et al.*, 2011) and 1/3 are entirely dependent (Rodger *et al.*, 2021). Pollinators use plants' conspicuous floral displays to inform foraging decisions when seeking food (Latty & Trueblood, 2020). Nectar has been the focus of bee research for reward and training because, as a simple solution of sugars, amino acids, and secondary metabolites (Egan *et al.*, 2018), it provides immediate feedback to foragers (Parachnowitsch *et al.*, 2019). Research on how nectar is used by foraging bees has provided fundamental insights into insect cognition, behaviour and learning (reviewed in (Giurfa, 2007; Leonard & Masek, 2014). However, little is known about how insects make decisions when foraging for pollen, the second most common pollinator reward.

Pollen is produced by all flowering plant species as the male gametophyte (Dajoz *et al.*, 1991), and is consumed by many species of bees, beetles, flies, wasps, moths and butterflies (Simpson & Neff, 1981; Kevan & Baker, 1983). While most plant species produce both nectar and pollen, 8-10% of flowering plants lack nectar and use pollen as their sole reward (i.e. buzz pollinated plants) (Buchmann *et al.*, 1983). Pollen is a rich source of protein and lipid which pollinators either consume directly or feed to offspring (Schulz *et al.*, 1998). Bees are particularly dependant on pollen as their primary protein source (Nicolson, 2011), and have evolved specialist morphologies and behaviours for its collection (Chapter Two; Renner, 1983; Thorp, 2000; Michener, 2007a; Hargreaves *et al.*, 2009; De Luca & Vallejo-Marín, 2013; Russell *et al.*, 2017).

Pollen and nectar have little nutritional overlap, playing different roles in the life cycles of pollinators (i.e., easily digestible sugars in nectar provide energy for adult locomotion while proteins and lipids in pollen are used for growth and development) (Palmer-Young *et al.*, 2019; Ruedenauer *et al.*, 2019; Nery *et al.*, 2020), and also follow divergent foraging rules (Konzmann & Lunau, 2014; Muth *et al.*, 2017). Yet how pollen foragers learn and behave in response to floral signals has been scarcely investigated. Bees will balance nutritional needs by adjusting foraging behaviours (Francis *et al.*, 2016) and choices (Leonhardt & Blüthgen, 2012; Vaudo *et al.*, 2016), so we would expect to find distinct behaviours adapted to maximise pollen foraging efficiency. Research has established that pollen foraging bees can select more rewarding flowers (Buchmann & Cane, 1989; Burkart *et al.*, 2014; Russell *et al.*, 2016a,b), but the mechanism underlying this has not been identified. It is also unclear whether bees respond to the presence, quality, or quantity of pollen (for a review see Nicholls & Hempel de Ibarra, 2017; Cook *et al.*, 2005; Arenas & Farina, 2012; Muth *et al.*, 2016a; Russell *et al.*, 2016a,b; Nery *et al.*, 2020). Alternatively, bees may not be able to assess pollen quality or quantity directly but rely instead on floral signals to make foraging decisions.

Bees use sight to make foraging decisions (Dobson, 2006; Dötterl & Vereecken, 2010; Aurore *et al.*, 2012). Learned associations between pollen and visual cues, such as colour, are used when pollen foraging (Lunau, 2000; Nicholls & De Ibarra, 2014; Muth *et al.*, 2015, 2016a), such as the colour of corollas and anthers (Muth *et al.*, 2016b). Bees have an innate preference for yellow (Raine & Chittka, 2007b; Ings *et al.*, 2009; Muth, 2021) even in non-foraging choices (i.e., play (Dona *et al.*, 2022)), which is likely due to the strong association of yellow with pollen (Lunau, 2000; Heuschen *et al.*, 2005). In fact, some plants use this innate bee-preference for yellow to secure pollination deceitfully by producing yellow coloured pollen-mimicking structures (Johnson *et al.*, 2003; De Luca & Vallejo-Marín, 2013; Russell *et al.*, 2020).

Scent is another key cue used by foraging bees (Raguso, 2004a). Honeybees can associate scent with pollen (Arenas & Farina, 2012), intrinsically responding to pollen scent (Nicholls & Hempel de Ibarra, 2013), and can use it to inform associative learning (Grüter *et al.*, 2008). However, bees do not immediately respond to pollen and continue learning while foraging, with pollen quality (e.g., chemical composition) affecting the likelihood of repeated visits to flowers (Muth *et al.*, 2016a). Scent not only influences bee attraction but can also trigger specialist foraging behaviours, such as pollen collecting by buzzing (i.e., buzz-pollination) (e.g., in *Solanum*; Chapter Two; Russell *et al.*, 2017, 2018). Despite recent interest, the role of scent in regulating pollen foraging behaviour remains poorly understood.

Here we investigated whether bumblebees use scent to make decisions when foraging for pollen. Specifically, we asked whether selection of artificial flowers offering only pollen as reward was influenced by scent and assessed how bees' preference changed during a single bout of foraging (i.e., a continuous period the bee spent seeking food outside the nest; Figure 3) depending on whether or not reward was paired with scent. Our hypotheses, that pollen foraging bees use scent to make foraging decisions and initially select flowers based on scent before learning to overcome initial preferences to select rewarding flowers, are supported by our results.

Methods

Experimental Design

We used a choice assay to explore whether pollen foraging bees use scent to make decisions, and how they respond when scent indicates an absence of rewards. Flower naïve bumblebee workers (*Bombus terrestris audax*) were presented with one of two experimental treatments: (1) Scent=Reward and (2) Scent≠Reward (Table 5). In the Scent=Reward treatment scent was paired

with a pollen surrogate while unscented flowers were unrewarding; in the Scent $\neq$ Reward treatment the reverse was true (Table 5). *Lycopodium* spores were chosen as the pollen surrogate as they are a granular, pollen-like organic powder which emits little scent (Table 2, Appendix 4). Scent treatments consisted of either 5 μ l linalool solution (1:100,000 linalool:hexane by volume) in “Scented” flowers, or the same volume of pure hexane in control, “Unscented” flowers applied to artificial flowers (Figure 19A and Figure 19B, explained below) and the hexane allowed to evaporate. Four artificial flowers of each condition (n=8) were randomly positioned in an 8 $\times$ 8 array (Figure 19C). Flowers visited by bees were immediately replaced with a flower of the same treatment in a random location and all flowers replaced between trials.

Table 5. Bees foraged on arrays of artificial flowers in which pollen was presented in scented flowers (Scent=Reward) or unscented flowers (Scent $\neq$ Reward). Individual bees were presented with flowers of one treatment only.

	Scented flowers	Unscented flowers
Scent=Reward Treatment	Rewarding	Unrewarding
Scent $\neq$ Reward Treatment	Unrewarding	Rewarding

Bee protocol

Commercially produced *Bombus terrestris audax* colonies (Biobest Group NV, Westerlo, Belgium) were maintained in the lab at 19-23°C and fed *ad libitum* Biogluc sucrose solution (Biobest Group NV, Westerlo, Belgium) inside the colony box. Ground honeybee-collected pollen (Agralan Ltd., Wiltshire, UK) was available in the flight arena outside experimental periods to acclimate pollen foragers to the arena. Plastic tubes connected colonies to the flight arena and access was controlled by a series of gates. To allow selection of motivated foragers, groups of 2 to 5 bees were released into the foraging arena. The first bee to interact with the experimental array was designated the focal bee and all others were captured and returned to the colony. If 10 minutes elapsed with no bee interacting with the array, then all bees were captured and returned. Focal bees freely foraged in the arena until they either re-entered the colony or had not interacted with a flower for five minutes, whichever occurred first. To allow future identification, all bees which had completed a foraging bout were captured, immobilised in a bee plunger, and marked with a unique number-colour code plastic disc glued to the thorax. Marked bees were returned to the colony.

Artificial flowers

Artificial flowers (Figure 19A and Figure 19B) were developed in a series of preliminary experiments (Appendix 12) and were designed to obscure bees' capacity to assess reward presence. Flower tubes (50mm long x 22mm diameter) were made by removing the bottom from 30ml screw-cap plastic tubes (Greiner Bio-One, Kremsmünster, Austria: product code 201150) and externally painted grey with two yellow "pollen guides" (c.5 mm wide) running straight from the mouth to the base of the tube (Flash Gitz Yellow, Citadel Colour). Corollas were yellow plastic discs (38mm diameter) and anthers consisted of 20mm long white pipe cleaner (Little Crafty Bugs Ltd., UK: product codes DE/FM-CPT80403 and CV/PC-51619, respectively). Pipe cleaners were pushed through the tube lid and hot glued in position. Tube lids were hot glued onto a wooden dowel (130mm x 4mm), painted grey, allowing horizontal flower presentation. During experiments, scent treatments were pipetted into the tube cap at the base of the corolla tube.

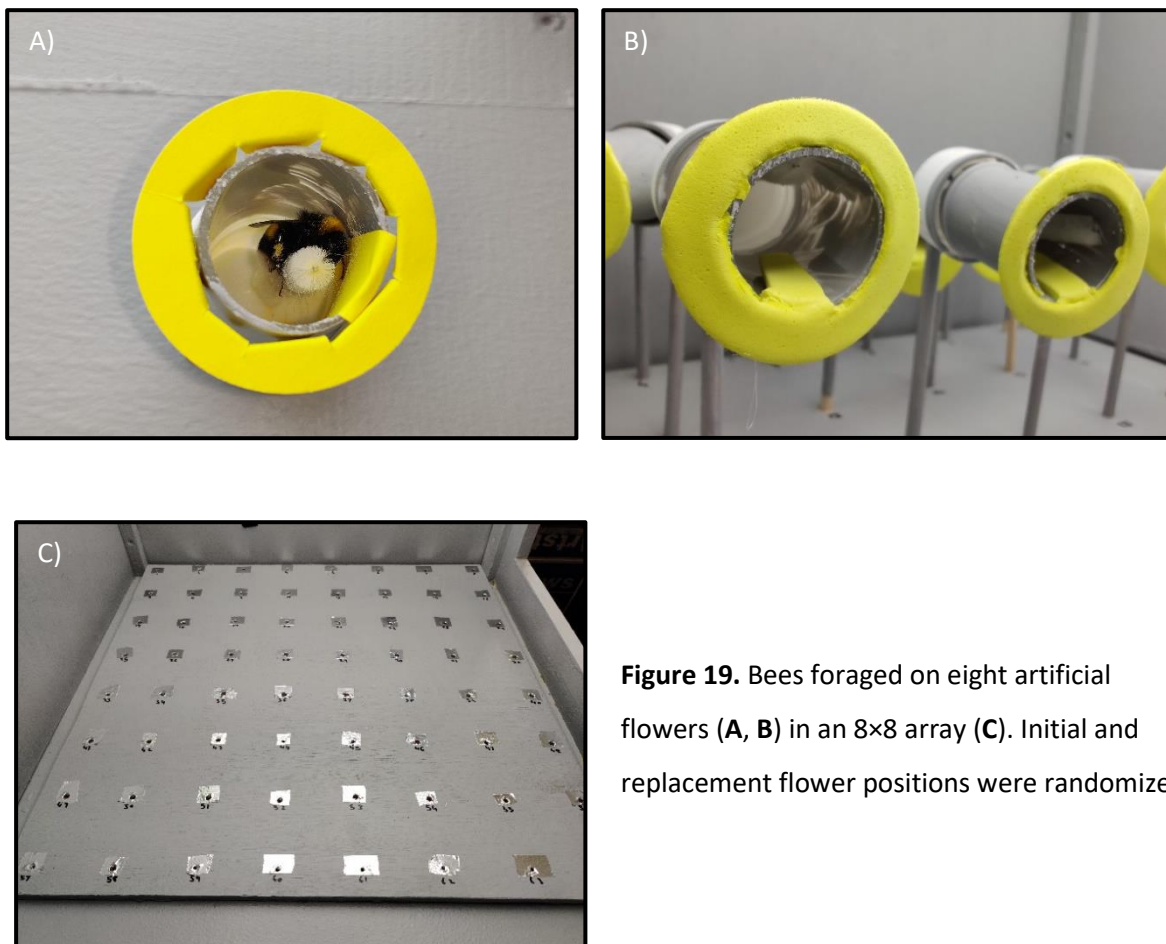


Figure 19. Bees foraged on eight artificial flowers (A, B) in an 8×8 array (C). Initial and replacement flower positions were randomized.

Flight arena

Experiments were done in a 70×50×25 cm (L×W×H) flight arena consisting of floor and 3 walls made of wood painted grey, a transparent acrylic ceiling and a flexible plastic mesh access panel.

Illumination was provided by an LED light panel (59.5 × 59.5 cm, 48W Daylight; Opus Lighting Technology, Birmingham, UK). Trials were filmed with a Sony Cyber-Shot DSC-RX100M7 (Sony Tokyo, Japan) camera outside the arena attached to an external microphone (H4n Pro Portable, Zoom, Tokyo, Japan) within the arena.

Data analysis

Data analysis and graph generation was done in R (R Core Team, 2022). We assessed two measures of bee decision making time, the time between floral visits and the time spent within a flower, using Generalised Linear Mixed Models (GLMM; (*glmer()* function, lme4 package, Bates *et al.*, 2015). In both models experimental treatment was fitted as a fixed effect, with bee identity, colony and visit as random effects (Decision Time ~ Experimental Treatment + (1 | Colony : Bee ID : Visit)). Assumptions were checked with the DHARMA package (Hartig, 2022) and stepwise model simplification of fixed effects undertaken.

We assessed how successful bees were at selecting rewarding flowers and how this changed through a foraging bout using a binomial GLMM (binomial distribution, logit linked). Bees' flower choice was coded as a binary response (rewarding flower = 1, unrewarding flower = 0) scent treatment (paired or unpaired with the reward) and choice number as fixed effects. Initially bee identity was nested within colony, however, as this estimated zero variance colony was dropped from the model (Flower Choice ~ Experimental Treatment * Visit Number + (1 | Bee ID)). Assumptions were checked with DHARMA package (Hartig, 2022) and a figure of model predictions (Figure 20) was produced by combining plots made in the ggplot2 (Wickham, 2016) and sjPlot packages (Lüdtke 2017; *plot_model()* function).

The likelihood of bees buzzing when visiting flowers in each treatment was analysed with a fully factorial binomial GLMM (buzzing visits coded = 1, non-buzzing visits = 0). Behavioural assessment of 22 visits was not possible due to problems with the AV recording, so 866 visits were analysed. Scent and visit were fitted as fixed effects. Again, colony identity was dropped from the nested random effect structure (Buzzing Behaviour ~ Treatment * Scent + Visit + (1 | Bee ID)). Visit was found to be non-significant so was dropped from the model, and assumptions were checked in DHARMA.

Results

In total 45 bees from 4 colonies completed 888 experimental visits. Seventeen bees completed the Scent=Reward treatment, making a mean of 16 floral visits (± 15.5 SD) each, while the 28 bees which completed the Scent $\neq$ Reward treatment made a mean of 17 visits (± 14.2 SD). The amount of time bees spent searching for flowers in the array was equal in both experimental treatments (GLMM: $P > 0.05$; Appendix 14A) and there was no difference in the amount of time bees invested in foraging within a flower (GLMM $P > 0.05$; Appendix 14B).

Scent influenced initial flower choice: scented flowers received 76% of bees' first visits across both treatments (86% when paired with reward, 68% when unpaired) (Figure 20, Appendix 13) and so bees were significantly more likely to initially choose rewarding flowers which were scented (GLMM, Treatment: $X^2_{(1)} = 21.3$, $P < 0.001$) (Figure 20, Table 6). However, bees' preference for rewarding flowers changed significantly the longer they foraged and the more flowers they visited. In the Scent=Reward treatment bees made fewer visits to rewarding flowers the longer they foraged (Visit: $X^2_{(1)} = 4.4$, $P = 0.035$), while bees in the Scent $\neq$ Reward treatment became more likely to choose rewarding flowers (Interaction: $X^2_{(1)} = 8.4$, $P < 0.005$) (Figure 20, Table 6).

All bees buzzed, with 40.9% of floral visits containing buzzes (Scent=Reward 17.4% of 403; Scent $\neq$ Reward 60.6% of 463). Bees buzzed the least when visiting flowers which contained neither reward nor scent (7.7% $\pm 2.1\sigma$ of visits), where only 2 bees buzzed. Bees were much more likely to buzz when visiting flowers presenting either scent, where buzzes occurred in 49.1% ($\pm 32.3\sigma$) of visits (GLMM: $X^2_{(1)} = 9.85$, $P < 0.005$), or reward where 51.3% ($\pm 32.6\sigma$) of visits contained buzzes ($X^2_{(1)} = 33.68$, $P < 0.001$) (Figure 21, Table 7). However, there was a significant negative interaction, with flowers presenting both scent and reward being buzzed in only 12.4% ($\pm 17.8\sigma$) of visits ($X^2_{(1)} = 9.85$, $P < 0.001$) (Figure 21, Table 7). Flowers which contained both reward and scent were more likely to be buzzed than those which contained neither (Appendix 16A; $X^2_{(1)} = 12.19$, $P < 0.001$). There was no significant change in the likelihood of bees buzzing as they gained experience ($P > 0.05$).

Table 6. Bees' flower preference was analysed with a binomial (logit) generalized linear mixed model, significant P-values are bold. Conditional R^2 (proportion of variance explained by the model) is 0.388, marginal R^2 (variance explained by fixed effects) is 0.107.

Model: Flower Choice ~ Treatment * Visit + (1 Bee Identity)					Analysis of Deviance (Type III Wald Chi Square tests)		
Fixed effects:	Estimate	Std. Error	z-value	P-value	X ²	Df	P-value
Intercept	1.680	0.367	4.58	<0.001	20.983	1	<0.001
Treatment	-2.095	0.454	-4.62	<0.001	21.313	1	<0.001
Visit	-0.025	0.012	-2.11	0.035	4.448	1	0.035
Visit * treatment	0.046	0.016	2.91	0.004	8.442	1	0.004

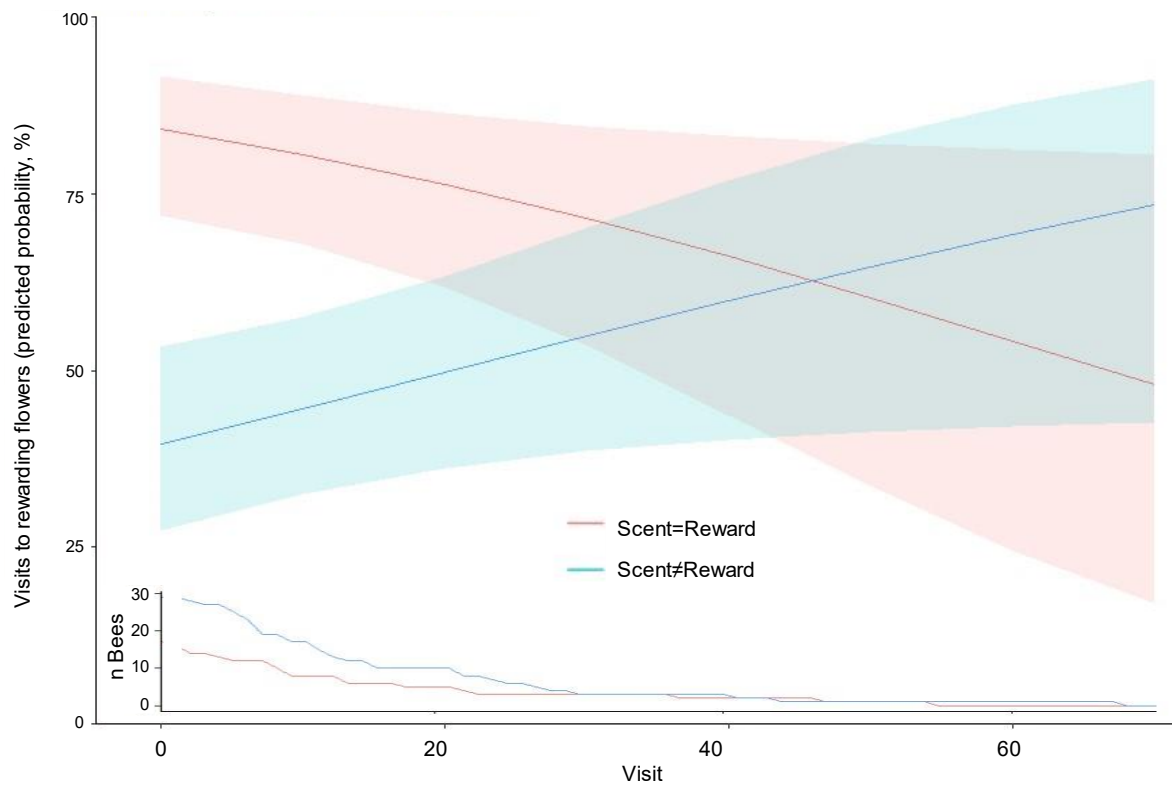


Figure 20. Bees initially used scent to select flowers and were significantly more likely to select rewarding flowers which were scented. However, as bees gained more experience, interacting with more flowers, their preference in both treatments changed significantly. In the Scent=Reward treatment likelihood of bees selecting rewarding flowers decreased while in the Scent≠Reward treatment bees became more successful at selecting rewarding flowers. Overlaid frequency histogram shows the number of floral visits bees made. Figure 20 presents the binomial GLMM model predictions and the raw data are presented in Appendix 13.

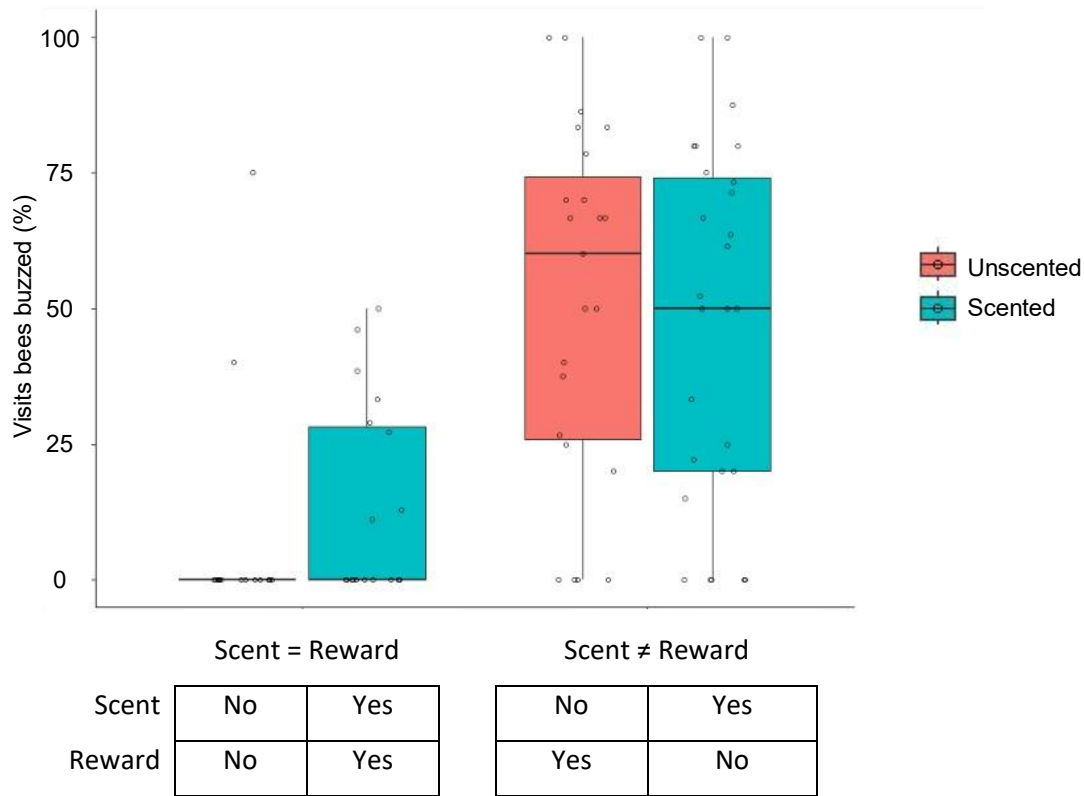


Figure 21. When reward and scent were paired bees preferentially buzzed on rewarding, scented flowers, with the likelihood of buzzing visits increasing as bees gained experience. There was no difference in buzzing visits when scent and reward were presented in different flowers. Appendix 16 presents the GLMM model predictions.

Table 7. The likelihood of bees buzzing when visiting artificial flowers was analysed with a binomial (logit linked) GLMM. Significant values are in bold. Conditional R^2 (proportion of variance explained the model) is 0.283, marginal R^2 (variance explained by fixed effects) is 0.510.

Model: <i>Buzzing Behaviour</i> ~ <i>Reward</i> * <i>Scent</i> + (1 <i>Bee ID</i>)					Analysis of Deviance Table (Type III Wald Chi Square tests)		
Fixed effects:	Estimate	Std. Error	z-value	P-value	χ^2	Df	P-value
Intercept	-3.58	0.58	-6.14	<0.001	37.68	1	<0.001
Reward	3.74	0.65	5.80	<0.001	33.68	1	<0.001
Scent	1.49	0.47	3.13	<0.005	9.85	1	<0.005
Scent * Treatment	-1.44	0.53	-2.70	<0.01	7.30	1	<0.01

Discussion

All angiosperms produce pollen which is a key part of bees' diets (Dajoz *et al.*, 1991; Thorp, 2000), yet how bees forage for this vital resource is poorly understood. We explored the role of scent in pollen foraging by presenting flower naïve bumblebees with artificial flowers where scent indicated either the presence or absence of reward. We found that pollen foraging bees used scent to select flowers, learning to stop using scents which prove unrewarding. Surprisingly, the amount of time bees spent searching was equal regardless of whether scent and reward were paired, and floral visit duration was equal in all conditions. These results suggest that bees detect pollen when foraging but pollen assessment is not immediate, resulting in relatively slow learning which may account for the abundance of pollen mimicking systems (Lunau, 2000, 2006), but scarcity of nectar mimicry (Lunau *et al.*, 2020).

Bees learn to use scent to find pollen

We found that scent informs flower selection by bees, with bees initially preferring scented flowers but learning to select unscented ones if scent does not indicate reward. Linalool is a well characterised pollinator attractant found in >50% of flowering plant families (Raguso & Pichersky, 1999; Knudsen *et al.*, 2006; Raguso, 2016), and we expected it to attract pollen seeking bees. Our experiment focused on exploring bees' response to scent when it occurred in flowers without pollen reward. Where scent and reward were unpaired, bees became more successful at finding rewarding flowers as they gained experience. Bees presented with scented rewarding flowers became more likely to visit unrewarding ones through a foraging bout, but this could be adaptive because apparent errors in a natural setting may lead to the discovery of novel food sources with long-term fitness benefits (Evans & Raine, 2014; Muth, 2021). Time spent within the nest appears important to bee learning (Chapter Two) perhaps allowing memory consolidation (Melnattur *et al.*, 2015) or reinforcement through social interaction (Molet *et al.*, 2009). Learning and memory retention over multiple foraging bouts may be a fruitful area of future research.

Flower search time was equal in length regardless of reward presence

Contrary to our expectation, that decision making would take longer for learning bees, the time between floral visits was equal regardless of scent and reward presence. This suggests the decision to land may be made during the final approach to flowers (Russell *et al.*, 2020) and that the time bees spend foraging is not exclusively allocated to selecting the next flower but taken up with other tasks such as pollen grooming or seeking other flower patches. Alternatively, flower replacement during experiments may disturb normal foraging behaviour. Why pollinators do not spend all their

time foraging, be it in a lab array or floral patch in the wild, visiting all available flowers before leaving a patch would be worth investigating.

Bees can sense pollen, but not immediately

Our observations of bee learning confirms that pollen foraging bees detect rewards, but the mechanism of how bees detect pollen remains debated (Nicholls & Hempel de Ibarra, 2017). The delay in learning suggests bees cannot sense pollen immediately. In fact, bees' failure to reject unrewarding flowers quickly, as observed in our experiment, where bees were spending equal time in all flower types, supports this. Touch undoubtedly plays a role and, without other sensing mechanisms, may explain observations of bees collecting inert granular pollen-like substances (i.e., glass beads, plastic granules, and cellulose powder) (Arenas & Farina, 2012; Konzmann & Lunau, 2014). Once collected, unwanted pollen can be groomed and discarded, instead of being stored in scopae, (Azo'o *et al.*, 2011; Lunau *et al.*, 2015) suggesting that quality assessment occurs post collection. This could be through tactile cues, chemo-receptors (Nicholls & Hempel de Ibarra, 2013; Pearce *et al.*, 2017), taste (Michener *et al.*, 1978; Michener, 2007b; Raine & Chittka, 2007a), or nutritional assessment via proxies such as pollen surface proteins or sugars (Leonhardt & Blüthgen, 2012; Ruedenauer *et al.*, 2019). Resolving this debate is important as it would allow more informed experimental design and have implications for pollinator health. For example, human activities which impair cognition (i.e., pollution McFrederick *et al.*, 2009 and use of pesticides Muth *et al.*, 2019; and herbicides Cullen *et al.*, 2019) would be expected to cause bees to make poorer foraging decisions, thus reducing fitness and increasing extinction risk.

Buzzing pollen collection was stimulated by both scent and reward

Over 10% of flowering plants are buzz-pollinated: floral vibration by bees is needed to release pollen (Buchmann *et al.*, 1983; De Luca & Vallejo-Marín, 2013). While this phenomenon is largely explored in the context of flowers dependant on buzzing due to morphological specialization, bees apply this behaviour widely to unspecialised flowers to efficiently gather pollen (for a non-exhaustive list, see Russell *et al.*, 2017). Scent blends extracted from flowers trigger floral buzzing (Chapter Two, Russell *et al.*, 2018), but this is the only example we are aware of a single compound trigger. That said, bees buzzed less than when presented with scents from buzz-pollinated flowers (Russell *et al.*, 2018). When presented alone, both reward and scent triggered buzzing in half of visits. Linalool's status as a near-ubiquitous floral scent (Raguso & Pichersky, 1999; Knudsen *et al.*, 2006; Raguso, 2016) precludes it from being the sole trigger of floral buzzing: this behaviour would be wildly more common in nature and the literature if it were. We therefore suggest that both olfactory and tactile

cues (i.e., pollen grains and specialised floral structures of buzz pollinated plants) play a role in triggering buzzing in bees, though both need further exploration.

As both pollen surrogate and scent trigger buzzing, we expected the presence of both cues to trigger an equal or greater buzzing rate, but perplexingly, our experiment showed that buzzing rate was lower when both cues were present (scent and reward presented together = 12% and separately c.50%). There are three explanations for this finding: technical, stochastic, or biological effect. Technical effects can be eliminated with confidence as the same experimental arena was used throughout, with experimental paradigms interspersed with one another and all possible post-experimental issues from data input to analysis thoroughly explored. Furthermore, sufficient data were collected and provided findings of high statistical significance, suggesting stochastic effects are unlikely. This leads us to conclude the effect is biological, but we cannot offer a biological explanation. As the experiment was not designed to explore the triggers for buzz pollination, bees did not encounter all treatment combinations. Therefore, an explicit exploration of the influence of olfactory and tactile cues on buzzing in bees could illuminate this surprising result. We suggest such an experiment could also consider the convergent morphology of buzz-pollinated flowers (i.e., reflexed petals and poricidal structures) (Vogel, 1978; Faegri, 1986; De Luca & Vallejo-Marín, 2013; Russell *et al.*, 2016a).

Future directions

Pollen foraging remains largely unexplored, despite the vital importance of pollen foraging on the fitness of plants and pollinators, with wider ranging ecological implications due to the role of plants as the primary producers in terrestrial habitats (Ollerton *et al.*, 2011; Rodger *et al.*, 2021). This work, and that of Russell & Papaj (2016) and Muth *et al.* (2017), provide methods to allow further exploration of pollen foraging and we propose three promising research directions (further developed in Appendix 17). First, use of multimodal signals by both emitters and receivers is an active area of research to which plant-pollinator interactions are well suited (Austin *et al.*, 2019) which could be investigated by studying bee responses to different rewards, and visual and scent cues. Secondly, honest signals (i.e., those which correlate with reward quality or quantity) are found in many foraging situations (Chapter Three) (Dobson *et al.*, 2000; Wright & Schiestl, 2009; Knauer & Schiestl, 2015; Gervasi & Schiestl, 2017; Burdon *et al.*, 2020) including pollen and nectar foraging which could be explored by training bees to associate visual and scent cues with reward before altering reward quality or quantity. Finally, foragers face a subtle type of signal honesty where food sources produce the same reward and signal but prove unreliable or unequally rewarding due to visitation by other foragers. Manipulation of reward quality, quantity and reliability after a training

period and observation of behavioural responses (i.e., flower switching, decision making time and foraging motivation) would allow exploration of how organisms react to uncertainty.

Conclusion

Our finding that bees learn to use scent when pollen foraging is not wholly surprising given that such associations have been found both in nectar foraging and wider foraging contexts. We also found that pollen foraging bees can learn to overcome initial preferences which prove unrewarding. Bees likely form many associations between sensory stimuli and rewards but how they learn to use these over a lifetime, and the possibly divergent rules pertaining to pollen rather than nectar collection should prove a clear avenue for future research. Further, our experimental design may provide the capacity to explore broader questions of floral constancy, multimodal signalling, reward uncertainty and honesty of signalling.

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Chapter Five. Pollination networks of plants with buzz-pollinated flowers in a tropical dry forest community (Yucatán, Mexico)

Abstract

Pollen consumption by floral visitors has led to the evolution of flowers with specialised morphologies which require buzzing for effective pollen removal. This excludes many clades of pollinators. Yet these buzz-pollinated flowers are often visited by inefficient pollinators, with negative consequences on plant reproductive fitness. These may be bees which buzz flowers but are the wrong size to effectively transfer pollen, or non-buzzing bees and other insects which gather pollen from flowers and may present specialisations to assist in pollen theft. Our aim was to describe the interaction networks of buzz-pollinated plants and their floral visitors. We studied five dry forest communities of buzz pollinated plants in Yucatán State, Mexico. Floral visitors were identified and their pollen collecting behaviours were classified (i.e., buzzing, or non-buzzing). This allowed us to ask the questions: (1) how do floral interaction networks vary between communities, (2) do floral visitor species use different pollen collecting behaviours when visiting different plant species, and (3) how do floral interaction networks differ if we consider either (a) all flower visitors or (b) only potential mutualists (i.e., buzzing bees). We found that buzz-pollinated flowers were almost exclusively visited by bees. However, most visits were made by non-buzzing bees, particularly eusocial species. All pollination networks were highly specialised, with species of floral visitors generally visiting few plant species, although buzz-pollinating bees were more likely than non-buzzing bees to visit multiple plant species. Bee species did not apply different pollen collecting behaviours to different plant species. This work highlights the importance of distinguishing mutualistic and non-mutualistic floral visitors when studying plant-pollinator interactions. Categorizing potential pollinators as mutualists or not will be more difficult for flowers with generalised morphologies than specialised buzz-pollinated ones, but it is critical for developing our understanding of this key ecological interaction in the face of global change.

Keywords

Pollination, interaction network, Solanaceae, Leguminosae, Asparagaceae, bee, fieldwork, buzz pollination, poricidally dehiscent anthers, neotropics.

Introduction

Animals are involved in the reproduction of over 80% of the world's ca. 369,000 species of flowering plants, with a third completely dependent on pollinators (Ollerton *et al.*, 2011; Darbyshire *et al.*, 2017; Rodger *et al.*, 2021). Although many of the diverse animals which visit flowers are effective pollinators, pollen collection is not undertaken altruistically. Pollen is a rich source of nutrients sought by floral visitors to consume directly or feed to their offspring, often constituting the pollinator's only source of protein and lipids (Roulston & Cane, 2000; Vaudo *et al.*, 2016; Steffan *et al.*, 2019). By eating the nutrient-rich pollen, pollinators come into direct conflict with plants: pollen is the male zygote of plants so this consumptive emasculation reduces plant's reproductive success through lost siring potential (Hargreaves *et al.*, 2009). In fact, less than 1% of pollen removed from flowers reaches a conspecific stigma (Harder & Johnson, 2008; van der Kooi *et al.*, 2021). This, therefore, creates strong selective pressure for the evolution of more efficient pollination through selection on floral traits (Harder & Routley, 2006) which is reflected in the geographic correlation between pollen limitation at the community scale and angiosperm species diversity (Vamosi *et al.*, 2006). One adaptation to reduce pollen loss to inefficient floral visitors, which has evolved independently multiple times, is the buzz-pollinated flower, found in over 22,000 species from 65 families (Vogel, 1978; Buchmann *et al.*, 1983; Faegri, 1986; Vallejo-Marín *et al.*, 2010; De Luca & Vallejo-Marín, 2013).

Buzz-pollinated flowers release pollen via apical pores or slits in response to vibrations (Buchmann *et al.*, 1983; Thorp, 2000), the production of which has only been observed in ca. 74 bee genera (ca. 58% of bee species) (Hedtke *et al.*, 2013; Cardinal *et al.*, 2018). Characteristically, a bee grasps the anthers with its legs and mouthparts, curves her abdomen and thorax over the pores or slits, and rapidly contract the indirect flight muscles without moving the wings to produce vibrations which expels pollen from the flower (Macior, 1964; Switzer *et al.*, 2016; Russell *et al.*, 2017). In this position effective pollinators collect pollen on their ventral side while also contacting the stigma, depositing pollen (Solís-Montero & Vallejo-Marín, 2017; Mesquita-Neto *et al.*, 2018, 2021). Here, any pollen consumed by the effective pollinator can be viewed as payment for services rendered.

Despite the role of the buzz-pollination syndrome in restricting pollen access, these flowers are still regularly visited by inefficient pollinators which are considered pollen thieves (Solís-Montero *et al.*, 2015; Solís-Montero & Vallejo-Marín, 2017; Mesquita-Neto *et al.*, 2018). Many bees with the capacity to buzz can effectively remove pollen but due to their size do not contact stigmas to deposit pollen (Carmo *et al.*, 2004; Díaz & Kitzberger, 2006; Hargreaves *et al.*, 2009; Solís-Montero & Vallejo-Marín, 2017). For example, small bees may buzz only a subset of anthers (Mesquita-Neto *et al.*,

2018, 2021). Buzz-pollinated flowers are also visited by insects which cannot buzz. These non-buzzing visitors fall into two general categories: active and opportunistic thieves (otherwise habitual or conditional thieves by Hargreaves *et al.*, 2009). Active thieves may have specialised adaptations to extract pollen or apply generalist tools to the task (Michener, 2007b). For example, *Trigona* bees are well documented chewing holes through anthers of buzz-pollinated flowers with their unspecialised mandibles to access pollen, while other bees may learn diverse behaviours to non-destructively remove pollen (Renner, 1983). Bees have evolved specialist structures for collecting pollen secreted within flowers (e.g., in tubular flowers or concealed anthers) including hooked hairs on legs or mouthparts, and barbed tongues (Thorp, 2000; Michener, 2007b). Conversely, opportunistic thieves take advantage of flowers by gleaning pollen which has been spilled by buzzing bees or gathering pollen from anthers damaged by active thieves (e.g., *Apis* spp.; Buchmann *et al.*, 1983).

In this study, we investigated the interactions between eight buzz-pollinated plant species and their floral visitors across five study sites in dry forest communities of Yucatán State, Mexico. We used focal plant observations to identify floral visitor species and classify whether they used floral vibrations to collect pollen, herein buzzing or non-buzzing insects (Renner, 1983; Mesquita-Neto *et al.*, 2018). We asked: (1) what are the differences between floral interaction networks across five different plant communities; (2) how does pollen collecting behaviour of floral visitor species change depending on which plant species they visit; and (3) how do floral interaction networks vary when we consider (a) only potential mutualists (i.e., buzzing bees) or (b) all flower visitors? Our findings provide novel insights on the proportion of legitimate and illegitimate floral visits in natural populations of buzz-pollinated plants and highlight future research directions.

Methods

Study area

Fieldwork was conducted from October-November 2019 in the tropical dry forest of Reserva Estatal Geohidrológica Anillo de Cenotes, Yucatán State, Mexico (Figure 22). The climate is tropical with a dry season from October-May and a wet season from June-September. Mean annual temperature is 27°C and rainfall is 1,000-1,200 mm (White & Hood, 2004; Dupuy *et al.*, 2012). The underlying geology is a flat and low-lying Cenozoic limestone plateau with thin soils and few rivers (White & Hood, 2004). The historic dominant ecosystem was the Yucatán dry forest, and though patches of primary forest survive, modern and historic human disturbance, namely cattle and henequen agriculture, and subsequent abandonment (Vaca *et al.*, 2012) have produced a mosaic of

Chapter Five. Pollination networks of plants with buzz-pollinated flowers in a tropical dry forest community (Yucatán, Mexico)

agricultural, scrub and secondary forest habitats. Managed pollinator populations are maintained in the Yucatán Peninsula and include the introduced *Apis mellifera*, and endemic stingless bees from the family Meliponinae (predominantly *Melipona beecheii* but also *Melipona yucatánica*, *Trigona fulviventrís*, *Nannotrigona perilampoides*, and *Cephalotrigona zexmeniae*) that have been managed for honey since pre-Colombian times (Bratman, 2020; Brown *et al.*, 2020).

Field sites

Five sites between 1.9–24.6 km apart where multiple species of co-flowering buzz-pollinated plants were found within ca. 50 m were selected (Figure 23, Table 9). Plantel Matilde is a block of closed canopy, secondary forest on abandoned agricultural land. San Isidro Ochil is an area of dense scrub with many semi-mature trees and limestone outcrops. Sotuta Peon North possesses a mixture of semi-mature secondary forest, open pasture, and open scrub (ca. 3:1:1 ratio). Sotuta Peon South consists of a 50:50 mix of mature forest and grazed pasture. X'Kanchackan is in a mosaic of dense and open scrubland with many semi-mature and mature trees and many limestone outcrops (Table 8). Of the sites, the habitat at Sotuta Peon South had the highest observed degree of human modification, and Plantel Matilde had the lowest.

Floral observations

To characterise pollination networks of buzz-pollinated plants we undertook focal plant observations to estimate floral visitation rates, to determine visitor identity and to categorise visitor behaviour. Buzz-pollinated plants were observed for 10 minutes, after which the observer moved to the next focal plant at the site until all plants had been observed and thereafter cycling continuously through the focal plants for the duration of the days' observations. Flying insects which landed on flowers of focal plants during observation had their pollen collecting behaviour classified and were caught with a hand net as they left flowers, killed with acetone and identified to species.

Table 8. List of study sites and duration of floral observations at each site in the Yucatán State, Mexico.

Site	North	West	Elevation (m)	Survey Days	Duration (minutes)
Plantel Matilde	20.837	89.440	21	4	1,837
San Isidro Ochil	20.648	89.343	2	2	364
Sotuta Peon North	20.752	89.564	17	2	479
Sotuta Peon South	20.740	89.764	18	2	379
X'Kanchackan	20.626	89.509	17	2	440

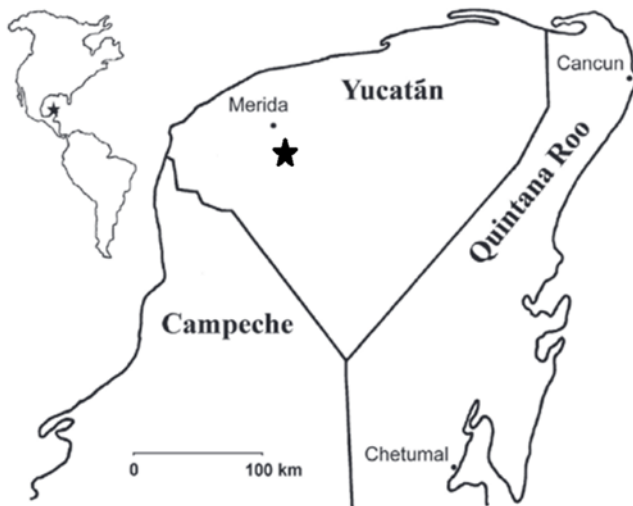


Figure 22. Fieldwork was conducted in Yucatán State, Mexico (Map from White & Hood, 2004), star indicates research area.

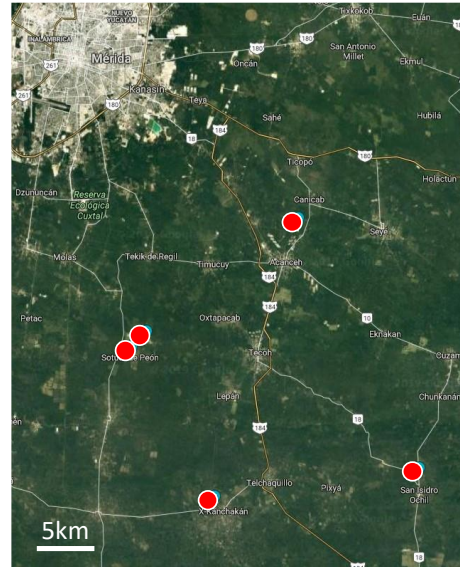


Figure 23. Five field sites were selected (red dots) where multiple species of buzz-pollinated plants were found co-flowering in Reserva Estatal Geohidrológica Anillo de Cenotes, South-East of Mérida, Yucatán State.

Behavioural classification: buzzing or non-buzzing?

As pollen is effectively expelled from buzz-pollinated flowers by vigorous vibrations, only floral visitors which can produce these vibrations can be effective pollinators. Therefore, flower buzzing insects were used as proxies for legitimate pollinators and any non-buzzing floral visitor was classified as a pollen thief. Therefore, the various pollen foraging behaviours seen were classified as either buzzing or non-buzzing. Buzzing behaviours were identifiable by two diagnostic features: the characteristic C-shaped posture of floral visitors (Figure 25A and Figure 25B) and the unmistakable, high-pitched acoustic component of buzzing. Floral visitors which buzzed when in contact with all of a flowers' anthers were classified as 'flower buzzing', while those which buzzed a subset of the anthers were classified as 'anther buzzing' (Figure 25).

Non-buzzing floral visits included the following foraging behaviours: 'anther milking' where pollen is removed by massaging pollen-containing anthers with mandibles in a basal-distal direction (Figure 25C); 'chewing' where anthers are bitten open (Figure 25D); 'gleaning' where pollen extracted by previous visitors is collected (Figure 25E); and 'tongue probing' where the tongue is inserted into the anther pore, or holes produced by chewing, to access pollen (not photographed).

Buzz-pollinated plant species descriptions

We observed floral visits to eight co-flowering buzz-pollinated angiosperm species from four genera representing three families. Anthers of these species dehisce poricidally (i.e., open via an apical pore), except *Capsicum annuum* which dehisce laterally (Table 9). These eight species were:

Solanum dasycanthum Brandegee (Solanaceae) is a small woody shrub with large broad-based prickles, reaching approximately 150 cm high (Figure 24A). The pentamerous, actinomorphic flowers lack nectar, are 10-15 mm in diameter, and are found in short unbranched inflorescences with one to two flowers and usually presented more or less horizontally. The deeply stellate corollas are white with a green basal star. Stamens are homomorphic, with five yellow, poricidally dehiscent, and connivent tapering anthers held in a central cone with short filaments. The round berries develop from green to red.

Solanum erianthum D.Don (Solanaceae) is a large shrub reaching approximately 350 cm high (Figure 24B). The pentamerous, actinomorphic flowers lack nectar, are 10-20mm in diameter held downward and presented on densely branching inflorescences of up to 50 flowers with many flowers co-flowering. The stellate corollas are white with a pale-yellow central star. Stamens are homomorphic, with five yellow, poricidally dehiscent, and connivent cylindrical anthers held in a central cone with short filaments. Spherical fruits develop from green to yellow and rapidly transition from pointing down to pointing up after fertilization.

Solanum houstonii Martyn (Solanaceae) is a small, dense woody shrub with sparse prickles reaching approximately 1.5m high (Figure 24C). The pentamerous, slightly zygomorphic flowers lack nectar, are 20-50 mm in diameter, grow on unbranched inflorescences, and are held horizontal to up-facing. Rotate corollas are purple with a yellow central star. Stamens are heteromorphic with poricidally dehiscent tapering anthers that vary from yellow to purple. The round berries develop from cream to pale green to black.

Solanum seaforthianum Andrews (Solanaceae) is an introduced woody vine native to tropical South America (Figure 24D). The species climbs up to 5m high into the low canopy. The pentamerous, actinomorphic flowers lack nectar, are 20-30 mm in diameter, are predominantly down-facing and grow in dense, branching inflorescences containing up to 50 flowers with many flowers open simultaneously. The stellate shaped corollas are purple. Stamens are homomorphic, with five yellow, poricidally dehiscent, and connivent cylindrical anthers held in a central cone with short filaments. The round berries develop from green to red.

Capsicum annuum L. var. *glabriusculum* (Dunal) Heiser & Pickersgill (Solanaceae) (Figure 24E) is a short shrub reaching around 120 cm high. The pentamerous, actinomorphic, down-facing flowers lack nectar, are 5-15 mm in diameter, and are born on small, branched inflorescences with rarely more than one open flower per inflorescences but many inflorescences per plant. The deeply stellate shaped corollas are white, with lobes spreading at anthesis. Stamens are homomorphic, with five deep purple, laterally dehiscent, and connivant cylindrical anthers held in a central cone with short filaments. Fruits are ellipsoid and apically pointed, green and held downward when developing, red and upwards pointing on stout pedicels when fully mature.

Senna occidentalis (L.) Link (Figure 24F) and *Senna lindheimeriana* (Scheele) H.S.Irwin & Barneby (Figure 24G) (Leguminosae) are both spreading herbaceous plants reaching up to 150 cm high. Their zygomorphic flowers are 20-40 mm in diameter, produce nectar and are held vertically in dense, unbranching inflorescences. Both species have five, free and bright yellow petals. Stamens are heteromorphic, with 7 longer and 3 shorter ones in each flower that differ in filament length; anthers are poricidally dehiscent and yellow to brown in colour, seed pods develop from green to black.

Echeandia luteola Cruden (Asparagaceae) (Figure 24H) grows from a basal whorl of leaves reaching up to 30 cm high, but flower spikes may reach 1 m. The hexamerous flowers lack nectar reward, are 8-12 mm in diameter, spaced ca.20 mm apart and born hanging pendulous from branched or unbranched lax inflorescences. Tepals are deeply stellate, pale yellow, and open in the afternoon with fully reflexed lobes, while fully enclosing the bright yellow anther cone at dawn (Appendix 18). Stamens are homomorphic, with six fused, yellow, poricidally dehiscent, tapering anthers held in a tight central cone with short, scaled filaments. Seeds are held in three lobed septicidal capsules which are green as seeds develop (visible in Appendix 18) turning brown at maturity as the capsule dries before splitting open from the apex.



Figure 24. Eight co-flowering buzz-pollinated plants found across the five field sites: (A) *Solanum dasyanthum*; (B) *Sol. erianthum*; (C) *Sol. houstonii*; (D) *Sol. seaforthianum*; (E) *Capsicum annum* var. *Glabriusculum*; (F) *Senna occidentalis*; (G) *Sen. lindheimeriana*; and (H) *Echeandia luteola*. (A-E) Solanaceae, (F-G) Leguminosae, and (H) Asparagaceae.

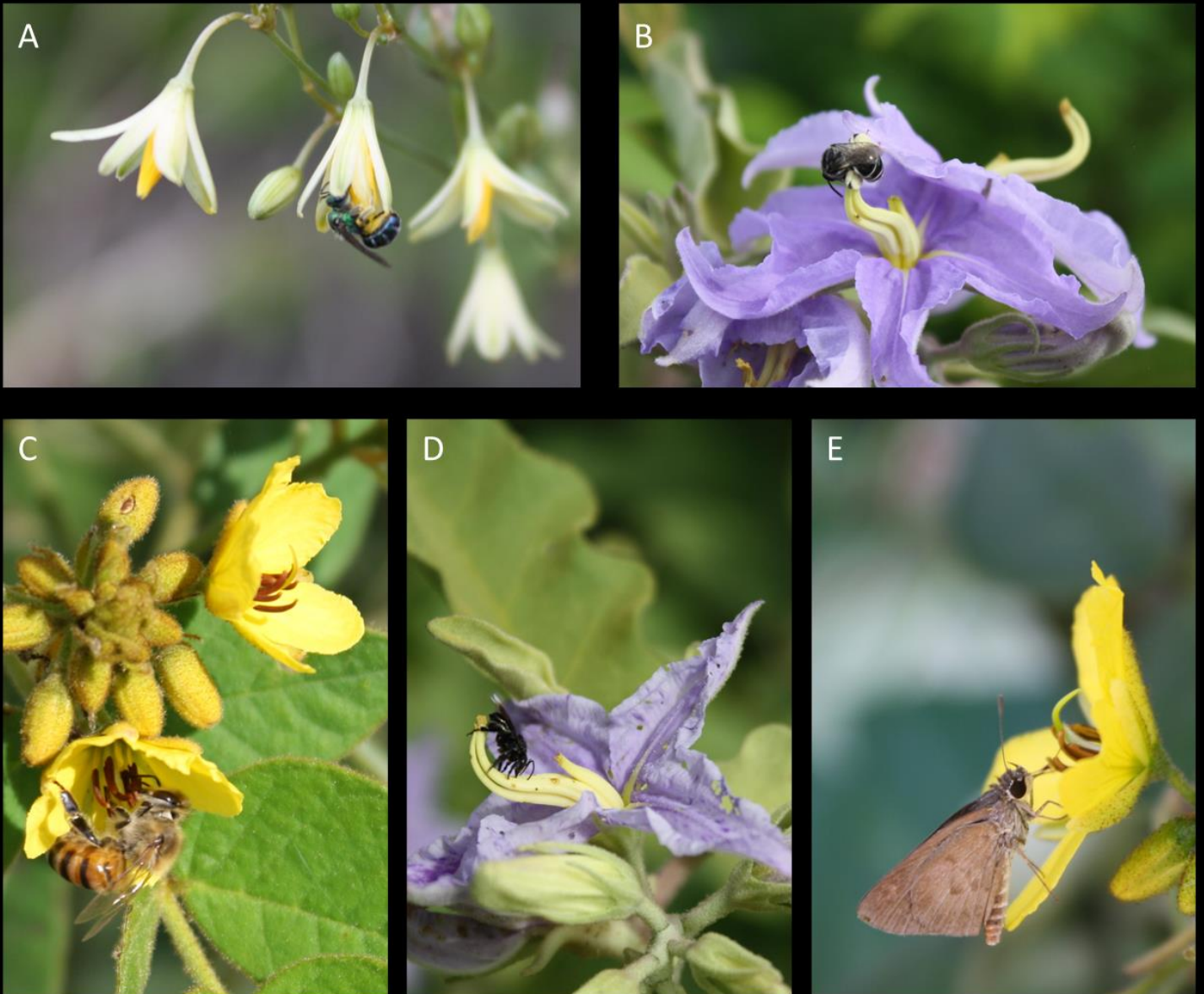


Figure 25. Behaviours observed in flower visits. Buzzing bees were considered as legitimate pollinators. (A) A halictid bee undertakes a flower buzzing visit on *Echeandia luteola*, where pollen is extracted via vibrations applied simultaneously to all the flowers' anthers. (B) Anther buzzing visit to *Solanum houstonii*, where the buzzing bee *Nomia tetrazonata* focuses on the longer anthers, leaving the shorter anthers untouched. Non-buzzing visits were classified as any floral visit which did not include floral buzzes and visitors considered pollen thieves (C-E). (C) Anther milking by *Apis mellifera* on *Senna occidentalis*, where the bee removes pollen from the anther pores by massaging anthers using its mandibles in a basal-distal direction. (D) *Frieseomelitta nigra* chewing through the anther wall of *Solanum houstonii*. (E) A butterfly on *Senna occidentalis* seeks nectar or gleans pollen (i.e., collects pollen extracted but not collected by a prior buzzing visitor). The final non-buzzing behaviour observed, tongue probing, occurred when floral visitors inserted their tongue into the anther pore to collect pollen (not photographed).

Table 9. The eight buzz-pollinated plant species were found across the five field sites.

Family	Species	Authority	Anther dehiscence	Plantel Matilde	San Isidro Ochil	Sotuta Peon N.	Sotuta Peon S.	X'Kanchakan
Solanaceae	<i>Solanum dasyanthum</i>	Brandegee	Poricidal	✓				
	<i>Solanum erianthum</i>	D. Don	Poricidal	✓		✓		
	<i>Solanum houstonii</i>	Martyn	Poricidal		✓	✓	✓	✓
	<i>Solanum seaforthianum</i>	Andrews	Poricidal	✓				
	<i>Capsicum annuum</i> var. <i>glabriusculum</i>	(Dunal) Heiser & Pickersgill	Longitudinal	✓				
	<i>Senna occidentalis</i>	L.	Poricidal		✓	✓	✓	✓
	<i>Senna lindheimeriana</i>	(Scheele) H.S.Irwin & Barneby	Poricidal	✓				
Asparagaceae	<i>Echeandia luteola</i>	Cruden	Poricidal		✓			

Data Analysis

As only 25 anther-buzzing visits were recorded in two sites, anther-buzzing and whole-flower-buzzing were pooled for data analysis, herein categorised as buzzing visits. Analysis and figure production was performed in R version 4.2.1 (R Core Team, 2022). To test whether the number of mutualistic, buzzing floral visits was related to the total number of floral visitors in a site we tested the relationship between the two using Spearman's Rank Correlation (`cor.test` function, *stats* package). Interaction networks were constructed using the *bipartite* package (Dormann *et al.*, 2009) as described by Dormann (2019). Interaction network plots were generated with the `plotweb` function of *bipartite*. The following network metrics were calculated for both the overall visitation network and the mutualistic, buzz-pollination sub-network (i.e., only considering buzzing visits) (networklevel function, *bipartite* package). **Niche Overlap** indicates the degree to which species exploit a set of resources, in this case bees use plants and *vice versa* (Pielou, 1972; Hurlbert, 1978). **Complementary Specialisation (H_2')** is a network-wide index of specialisation based on the deviance between the total possible number of species interactions and the observed number (Blüthgen *et al.*, 2006) with values ranging from zero (no specialization) to one (perfect specialization). **Weighted**

NODF (Nestedness Metric Based on Overlap and Decreasing Fill) is a measure of how nested a network is, varying from zero (perfectly non-nested) to 100 (perfectly nested) (Almeida-Neto & Ulrich, 2011). **Connectance** is a measure of network specialization representing the ratio of the number observed interactions to the number of possible interactions, whereby the more specialized the network, the lower the connectance value (Gotelli & Graves, 1997). **Links per Species** is the mean number of interactions between species in a network. **Alatalo Interaction Evenness** is a measure of habitat diversity interaction i.e., how balanced the distribution of interactions is across species, so as a habitat's diversity increases the closer to one (from zero) the value becomes (Alatalo, 1981; Müller *et al.*, 1999).

Direct comparison between the overall visitation and buzz-pollination sub-networks is not possible as they are inherently of different sizes (Bascompte, 2009; Losapio *et al.*, 2019). Therefore, network metrics were standardised using z-scores (from 10,000 randomized null-models for each network and sub-network), allowing comparison of networks of different sizes (nullmodel function, *bipartite* package; r2dtable null model method) which were used to calculate the deviance of observed network metrics from random expectation by calculating 95% confidence intervals (CI) and Z-scores (as in Losapio *et al.*, (2019) and Mesquita-Neto *et al.*, (2018) respectively). Z-Scores were calculated as $Z = \frac{\text{observed metric} - \text{null mean}}{\text{nulls standard deviation}(\text{null mean})}$ (Losapio *et al.*, 2019). Observed metrics were significantly non-random if they fell without the 95% CIs or had a Z-score greater than 2 (positive or negative).

Results

Summary of Observations

We observed plant-pollinator interactions of eight buzz-pollinated plant species across five communities over 16 days totalling 58.31 hours of observations. Flower visits commenced at 06:50 and peaked around 10:00. However, flowers of *E. luteola* did not begin to open until 14:00 and the two observed floral visits occurred at 15:35 and 16:05 (Appendix 18); despite this low visitation rate, all plants on site (focal and non-focal) had maturing seed pods. In total 422 floral interactions were observed and classified as non-buzzing (337 visits), flower-buzzing (53 visits) and anther buzzing (25 visits) (Figure 25,

Figure 26). There was no correlation between the total number of visits and the number of mutualistic visits (i.e., flower-buzzing bees) across the five field sites (Spearman's Rank Correlation: $r_s = -0.1$, $S_{(4)} = 22.0$, $P > 0.05$).

All flower-visiting bees were captured and identified to 31 morphospecies (Table 10) from four families (*Apidae*, *Colletidae*, *Halictidae*, and *Megachilidae*). Bees of the tribe *Meliponini* were the most abundant floral visitors (especially *Trigona* sp., *Trigona fulviventris*, *Frieseomelitta nigra*, and *Nannotrigona periamplodes*), none of which buzzed when visiting flowers. Flower-buzzing bees were less common, the most abundant of which were *Nomia tetrazonata*, *Euglossini* Sp., and *Halictidae* Sp.8. The pollen collecting behaviour of all observed bee species was consistent regardless of the plant species they visited, no behavioural shifting was recorded. During our observations just 3 non-hymenopteran insects were observed landing on flowers, all dipterans, which were excluded from analysis. Lepidoptera occasionally visited flowers of *Senna* Sp. (as in Figure 25), but never during observations of focal plants.

Plant-pollinator interaction networks

Two plant species were present at Sotuta Peon South (*S. houstonii* and *S. occidentalis*). Here, *S. occidentalis* received only one visit during observations from the non-buzzing *A. mellifera*, so no buzz pollination sub-network could be calculated (

Figure 26D, Figure 27D). Although the greatest number of plant-pollinator interactions were observed at Sotuta Peon South (n=148) only one buzzing visit was observed, from *Euglossa viridissima*: the lowest number and percentage of buzzing visits (n=1 and 0.7%). Furthermore, the buzz pollination sub-network at Sotuta Peon North was binary (consisting of two plant and pollinator species) with 10.2% of 59 interactions from buzzing bees (

Figure 26C) and so the network was not suitable for null-model generation. The greatest number of buzzing interactions (n=14) were seen at Plantel Matilde and X'Kanchakán where they comprised 20% and 15.5% of interactions, respectively; while San Idrisio Ochil had the greatest proportion of buzzing interactions (46.4% of 28 visits).

The degree to which species share resources, **Niche Overlap**, was lower for plants than for pollinators at all sites across both the overall network and the buzz-pollination sub-network. This indicates that plants were visited by many pollinator species, as could be expected when there are more pollinators than plants in a network, but pollinators rarely visited multiple plants (Table 11). Pollinator niche overlap was significantly lower than would be expected under a random visitation (Z-score < -2.0) in the overall networks of three sites (Plantel Matilde, Sotouta Peon South, and X'Kanchackan). Of these, the degree of overlap decreased in the buzz-pollination sub-networks of Plantel Matilde and X'Kanchackan and remained significantly lower than expected (Z-score < -2.0). Plant niche overlap was significantly lower than expected in all overall networks (Z-score < -2.0) and in the buzz pollination sub-networks at Plantel Matilde, San Isidro Ochil and X'Kanchakan.

The extent to which the number of observed interactions in a network deviates from the total possible number of interactions, **Complementary Specialisation (H_2')**, was higher than expected in all overall networks (Z -score <2) (Table 11) and the buzz-pollination sub-networks at Plantel Matilde and X'Kanchackan.

Overall, visitation networks were significantly less nested (lower **Weighted NODF** scores) than expected at all sites (Z -score <2) except Sotuta Peon North (Z -score = -0.32) (Table 11). Network nesting in buzz-pollination sub-networks was not different from the null models.

Networks were significantly more specialised (i.e., with lower **Connectance** scores) than predicted by the null models for the overall network at all sites (Z -score <2.0) except Sotuta Peon North where connectance was within expectations (Z -score = -0.10). The buzz-pollination sub-network at Plantel Matilde was also significantly more specialised than expected.

The mean number of interactions a species had with species of the other trophic level (**links per species**) was close to one across all networks, with fewest links at Sotuta Peon South (0.8) and most at Sotuta Peon North (1.44) (Table 11). Observed links per species was lower than expected at all sites except Sotuta Peon North ($Z > -2.0$), but only remained lower than expected in the buzz pollination sub-network of Plantel Matilde.

The diversity of interactions in a habitat, or the evenness of interactions across species, (**Alatalo interaction evenness**) was highly variable between sites (Table 11). At Plantel Matilde, Sotuta Peon North and X'Kanchackan the score for the overall network was no different than the null-models, while the score was lower than expected at San Isidro Ochil and higher at Sotuta Peon South. No buzz-pollination sub-networks were different from expected.

Table 10. Morphospecies of visitors to buzz-pollinated flowers across all dry forest field sites in Yucatán State, Mexico. Two categories of behaviours were recorded: buzzing and non-buzzing. Two types of flower buzzing behaviours were recorded: flower buzzing, where all anthers were contacted, and anther buzzing, where a subset of anthers were contacted. Three non-buzzing behaviours were observed (chewing, gleaning, and tongue probing), see Figure 25 for illustrations. These data are displayed graphically in

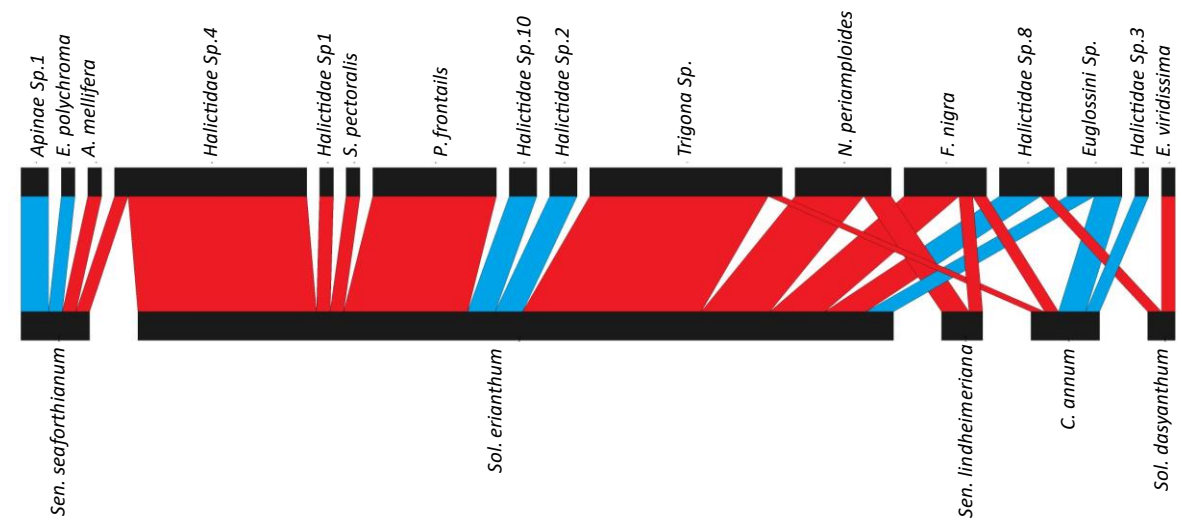
Figure 26 and Figure 27.

Family	Tribe	Morphospecies	Authority	Behaviour	n
<i>Apidae</i>	NA	<i>Apidae</i>	Latreille	Flower Buzzing	3
	<i>Apini</i>	<i>Apis mellifera</i>	Linnaeus	Non-Buzzing: Glean, Tongue Probe	16
		<i>Exomalopsis</i> Sp.1		Flower Buzzing	2
		<i>Exomalopsis</i> Sp.2		Flower Buzzing	1
		<i>Apinae</i> Sp.1		Flower Buzzing	2
	<i>Euglossini</i>	<i>Eulaema polychroma</i>	Mocsáry	Flower Buzzing	1
		<i>Euglossa viridissima</i>	Bembé & Eltz	Flower Buzzing	3
		<i>Euglossini</i> Sp.		Flower Buzzing	9
	<i>Meliponini</i>	<i>Cephalotrigona zexmeniae</i>	Schwarz	Non-Buzzing	1
		<i>Frieseomelitta nigra</i>	Cresson	Non-Buzzing: Tongue Probe, Chewing	35
		<i>Nannotrigona periamplodes</i>	Cresson	Non-Buzzing: Tongue Probe, Glean	34
		<i>Plebia frontalis</i>	Friese	Non-Buzzing: Tongue Probe	23
		<i>Scaptotrigona pectoralis</i>	Dalla Torre	Non-Buzzing: Tongue Probe	1
		<i>Trigona fulviventris</i>	Guérin-Méneville	Non-Buzzing: Tongue Probe, Glean, Chewing	80
		<i>Trigona</i> Sp.		Non-Buzzing	117
	<i>Xylocopini</i>	<i>Xylocopa mexicanorum</i>	Cockerell	Flower Buzzing	3
<i>Colletidae</i>		<i>Colletidae</i> Sp.1		Flower Buzzing	2
		<i>Colletidae</i> Sp.2		Flower Buzzing	2
		<i>Hylaeus</i> Sp.		Non-Buzzing: Gleaning	1
<i>Halictidae</i>		<i>Halictidae</i> Sp.1		Non-Buzzing: Tongue Probe	2
		<i>Halictidae</i> Sp.2		Flower Buzzing	2
		<i>Halictidae</i> Sp.3		Flower Buzzing	1
		<i>Halictidae</i> Sp.4		Non-Buzzing: Tongue Probe	14
		<i>Halictidae</i> Sp.5		Anther Buzzing	2
		<i>Halictidae</i> Sp.6		Anther Buzzing	1
		<i>Halictidae</i> Sp.7		Anther Buzzing	4
		<i>Halictidae</i> Sp.8		Flower Buzzing	8
		<i>Halictidae</i> Sp.9		Flower Buzzing	1
		<i>Halictidae</i> Sp.10		Flower Buzzing	2
		<i>Halictidae</i> Sp.11		Flower Buzzing	3

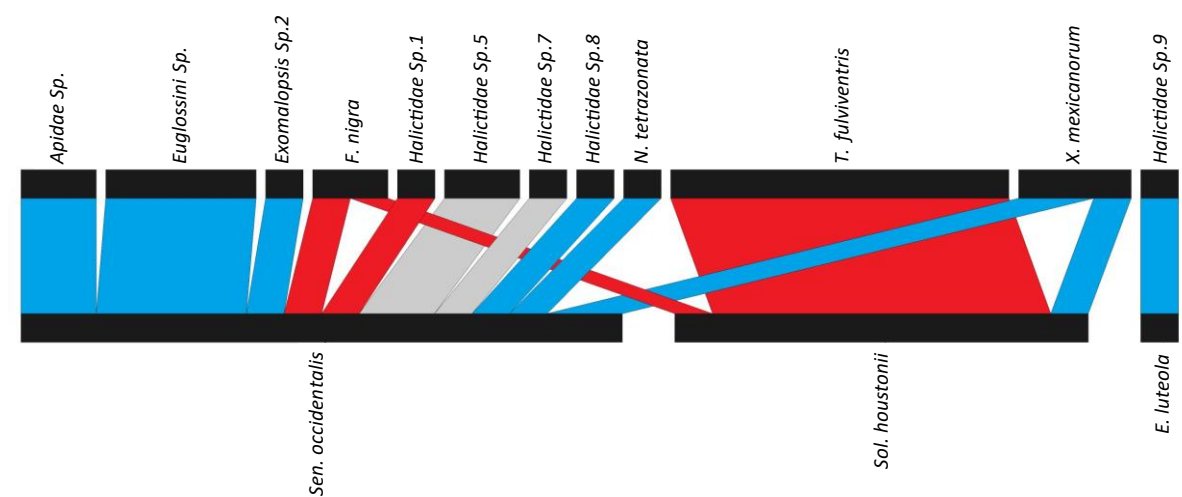
Chapter Five: Pollination networks of plants with buzz-pollinated flowers in a tropical dry forest community (Yucatán, Mexico)

	<i>Nomia tetrazonata</i>	Cockerell	Flower Buzzing	20
<i>Megachilidae</i>	<i>Megachilidae</i> Sp.		Non-Buzzing	1

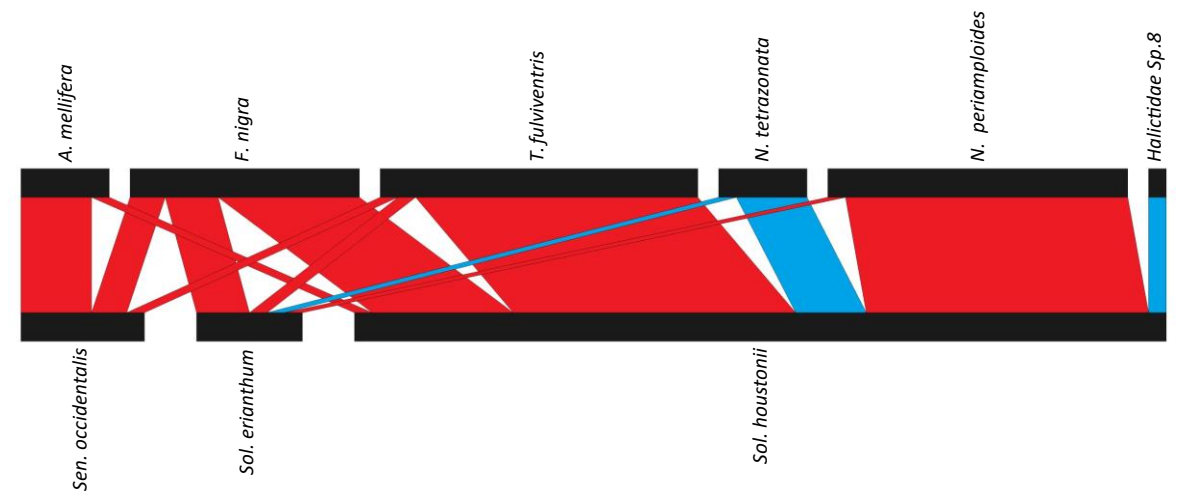
A) Plantel Matilde, n=70



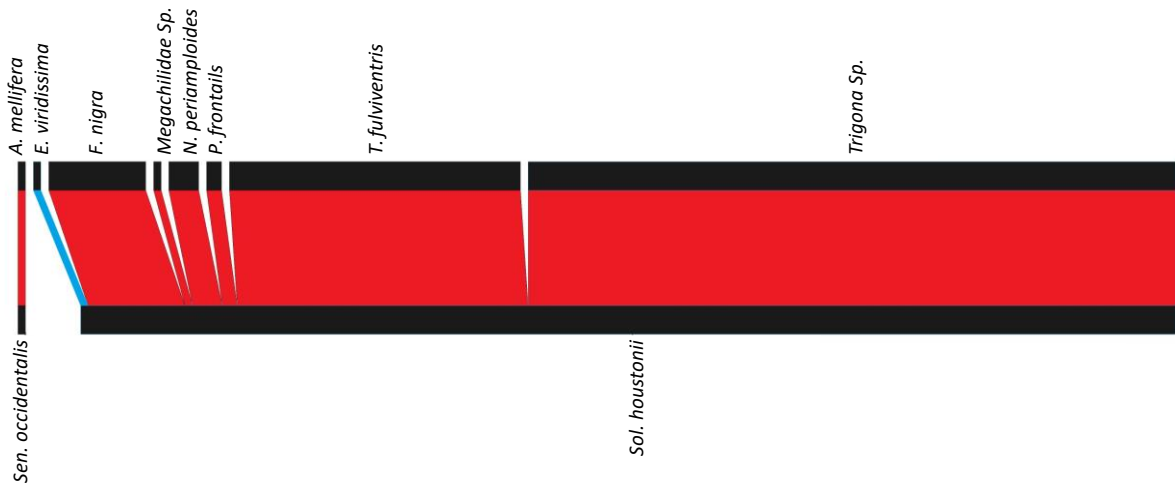
B) San Isidro Ochil, n=28



C) Sotuta Peon North, n=59



D) Sotuta Peon South, n=148



E) X'Kanchackan, n=90

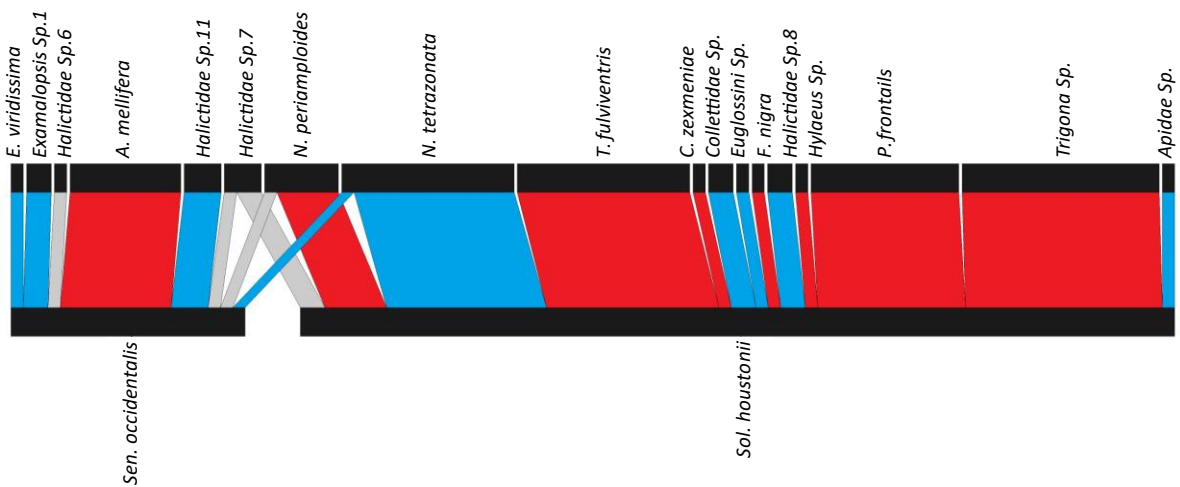


Figure 26. Visitation networks showing interactions between plants with buzz-pollinated flowers (bottom row of black rectangles) and floral visitors (top row of black rectangles) at each site. Colours indicate proportions of floral visits undertaken by bees classified as whole flower buzzing (blue), anther buzzing (grey) or non-buzzing (red).

Table 11. Visitation network metrics for the overall network and buzz-pollination sub-network for buzz-pollinated flowers. Z-scores >2 (positive or negative), in bold, indicate metrics significantly different from random chance (based on 10,000 randomised null models). Metrics were considered significant if they fell outside the 95% confidence intervals (CI) generated from the null models.

Network Metric	Visitation Network				Buzz Pollination Sub-Network			
	Null Model		Observation		Null model		Observation	
	95% CI Lower	95% CI Upper	Value	Z-Score	95% CI Lower	95% CI Upper	Value	Z-Score
Plantel Matilde								
Pollinator Niche Overlap	0.56	0.93	0.45	-3.12	0.27	0.57	0.22	-2.50
Plant Niche Overlap	0.21	0.54	0.10	-3.03	0.13	0.43	0.11	-2.10
H ₂ '	0.04	0.34	0.51	4.39	0.00	0.57	0.64	2.69
Weighted NODF	24.7	51.3	21.7	-2.44	0.00	18.1	10.3	0.58
Connectance	0.31	0.38	0.29	-3.10	0.34	0.47	0.31	-2.84
Links Per Species	1.19	1.43	1.10	-3.10	0.92	1.25	0.83	-2.84
Alatalo interaction evenness	0.62	0.73	0.66	-0.46	0.80	0.97	0.92	-0.01
San Isidro Ochil								
Pollinator Niche Overlap	0.47	0.70	0.64	0.99	0.58	0.95	0.75	1.00
Plant Niche Overlap	0.17	0.61	0.01	-2.55	0.00	0.50	0.08	-2.54
H ₂ '	0.07	0.51	0.81	4.77	0.00	1.00	0.54	0.45
Weighted NODF	11.6	33.5	7.7	-2.46	0.00	35.9	12.8	-0.01
Connectance	0.42	0.53	0.39	-2.59	0.33	0.41	0.37	-0.55
Links Per Species	1.00	1.27	0.93	-2.59	0.75	0.92	0.83	-0.55
Alatalo interaction evenness	0.68	0.85	0.64	-3.12	0.80	0.90	0.85	-0.28
Sotuta Peon North ¹								
Pollinator Niche Overlap	0.64	0.97	0.73	-1.22	NA	NA	NA	NA
Plant Niche Overlap	0.51	0.91	0.49	-2.45	NA	NA	NA	NA
H ₂ '	0.02	0.25	0.31	3.04	NA	NA	NA	NA
Weighted NODF	23.6	69.4	50.0	-0.32	NA	NA	NA	NA
Connectance	0.61	0.83	0.72	-0.10	NA	NA	NA	NA
Links Per Species	1.22	1.67	1.44	-0.10	NA	NA	NA	NA
Alatalo interaction evenness	0.67	0.80	0.69	-1.20	NA	NA	NA	NA
Sotuta Peon South ²								
Pollinator Niche Overlap	0.92	1.00	0.75	-6.82	NA	NA	NA	NA
Plant Niche Overlap	0.01	0.82	0.00	-2.01	NA	NA	NA	NA
H ₂ '	0.00	0.75	1.00	4.41	NA	NA	NA	NA
Weighted NODF	0.00	27.6	0.00	-4.70	NA	NA	NA	NA
Connectance	0.56	0.56	0.50	-7.11	NA	NA	NA	NA
Links Per Species	0.90	0.90	0.8	-7.11	NA	NA	NA	NA
Alatalo interaction evenness	0.62	0.64	0.64	4.17	NA	NA	NA	NA
X'Kanchackan								
Pollinator Niche Overlap	0.65	0.93	0.59	-2.77	0.57	0.52	0.50	-2.50
Plant Niche Overlap	0.59	0.92	0.07	-3.95	0.52	0.95	0.27	-4.89
H ₂ '	0.04	0.27	0.81	11.22	0.00	0.41	0.63	4.30
Weighted NODF	28.9	47.2	13.23	-5.50	11.0	30.2	13.79	-1.64
Connectance	0.64	0.78	0.58	-3.95	0.62	0.81	0.63	-1.51
Links Per Species	1.15	1.40	1.05	-3.95	1.00	1.30	1.00	-1.51
Alatalo interaction evenness	0.69	0.77	0.72	-0.38	0.53	0.77	0.57	-1.30

¹ Buzz-pollination network was binary, consisting of 2 plants and pollinators, so networks were not calculated.

² Buzz-pollination network contained 1 observation so metrics could not be calculated.

Discussion

Despite the widespread occurrence of pollen theft, the degree to which flowers are visited by pollen thieves is rarely studied at the community scale (Mesquita-Neto *et al.*, 2018, 2021). Buzz-pollinated plants present an ideal model system for studying pollen theft due to the audible buzzing sound produced by legitimate floral visitors. Contrary to the hypothesis that buzz-pollinated flowers evolved to exclude inefficient pollinators (De Luca & Vallejo-Marín, 2013), buzzing bees were the minority of floral visitors. Pollination networks were generally similar between our five field sites when considering all plant-pollinator interactions. However, the sub-networks considering only buzz-pollinating bees were highly variable.

Overall visitation network structure

Our results show that the overall visitation network at each site was more specialized than expected from the null model of no network structure (**plant niche overlap** and complementary specialisation; H_2') and, except at Sotuta Peon North, networks were less nested (**Weighted NODF**), less connected (**Connectance**) and contained fewer **links per species** than expected. Together these results demonstrate that bee species visiting buzz-pollinated flowers generally specialise, foraging on one plant species, rather than generalising across species with similar floral morphologies. This high degree of specialization may be due to divergent flower morphologies (Nunes *et al.*, 2021) regulating pollen access, which can be demonstrated by comparing *Solanum houstonii* and *Senna occidentalis*, present together in four sites. *Sol. houstonii* has much longer, more robust anthers, and more pronounced anther dimorphism than all other flowers while *Sen. occidentalis* anthers are thinner and finer, with specialised petals playing an active role in pollen deposition (Amorim *et al.*, 2017). Another mechanism with a role in structuring pollination networks are floral signals (Junker *et al.*, 2010; Junker & Blüthgen, 2010; Kantsa *et al.*, 2018; Kemp *et al.*, 2019, 2022). As floral scents play a key role in triggering buzzing pollen collecting behaviour in bees (Chapter Three, Chapter Four) (Russell *et al.*, 2018) we propose these are of particular interest for further investigation. Such research could be descriptive, mapping floral scent compounds against network structure, or more manipulative, experimentally altering floral scent and observing the changes in pollinator visitation and behaviours.

Buzz-pollination sub-networks

Across the available buzz pollination sub-networks (Plantel Matilde, San Isidro Ochil and X'Kanchackan), we found that the degree of **plant niche overlap** remained lower than expected but was marginally higher than in the overall pollination network. **Pollinator niche overlap** was also

lower than expected, and networks remained more specialised (greater H_2' values) in the buzz pollination sub-networks of Plantel Matilde and X'Kanchackan, arguably the least disturbed field sites. Together these findings suggest that buzzing bee species visit more buzz-pollinated plant species than non-buzzing bees. We hypothesise that this is because bees able to produce floral buzzes specialise in visiting plants which require buzz-pollination, applying this behaviour to multiple plant species. Conversely, the majority of floral visits were made by non-buzzing bees (77%) which may only visit flowers from which they have learnt to extract pollen or which they are morphologically adapted to exploit. This hypothesis could be tested through pollen load analysis of buzzing and non-buzzing floral visitors, as we would expect buzzing bees to carry more pollen from buzz-pollinated plants than non-buzzing bees.

Between site differences

Too few buzzing bees visited focal plants to calculate the sub-network at two sites (Sotuta Peon North and South). Although these sites had neither the least survey time, the fewest plants, nor fewest observed interactions (indeed the greatest number of plant-pollinator interactions were recorded at Sotuta Peon South), they were closest to one another, only 1.9km apart, and shared two of their three focal plant species. It is therefore possible that the pollination networks of both sites were shaped by the same landscape-scale ecological factors. For example, visitation of buzz-pollinated plants is impacted by landscape resource availability (Kemp *et al.*, 2022). As high turnover of resources and bee species is common between field sites and through time (Junker *et al.*, 2010), it is possible that plant-pollinator links were missed and we can reasonably expect that increased data collection would reveal plant-pollinator interactions overlooked here. However, relatively low visitation rates by buzz-pollinating bees means using the same methods would require considerable time investment. We therefore propose that, to identify or exclude the existence of additional plant-pollinator interactions, future data collection includes assessment of floral visitor pollen loads using palynology (Lando *et al.*, 2018) or metabarcoding (Bell *et al.*, 2017). How buzz-pollinated plants nest within the wider ecosystem's pollination network could be explored by additional surveys of the broader floral community.

Floral visitors of buzz-pollinated flowers

Buzz-pollinating bees made less than a quarter of the observed visits (23%). Halictidae was the most important tribe of legitimate visitor, being both most speciose and performing the most buzzing visits, as found elsewhere in the neotropics (Lando *et al.*, 2018 and references 29-31 within). Indeed, we observed all but two morphospecies of Halictidae buzz pollinating. *Nomia tetrazonata* (of the Halictidae) was the most common buzzing bee (26% of floral buzzes; present at San Isidro Ochil,

Sotuta Peon North and X'Kanchackan). The fact that potentially legitimate pollinators made up such a small proportion of floral visits was unexpected as the evolution of buzz-pollinated flowers is hypothesised to exclude inefficient pollinators (Harder & Barclay, 1994; De Luca & Vallejo-Marín, 2013) and contrasts with the findings of Mesquita-Neto *et al.* (2018). However, more detailed measurements of pollen theft and reproductive success at the ecosystem level would be required to draw conclusions on the evolutionary success of buzz-pollination. Furthermore, analysis of resource exploitation by buzzing bees beyond buzz-pollinated flowers, e.g., through assessment of bee pollen loads (Gonçalves & Buschini, 2017), and seasonal variation in visitation rates (Gérard *et al.*, 2020; Duchenne *et al.*, 2020) could reveal what plants buzz-pollinating bees prefer to visit in this environment.

Melipona beecheii is a buzz-pollinating bee endemic to Central America and popular with apiculturists; it has been managed for honey production in the Yucatán since at least the Mayan Classical Period (ca. 250-900CE) (Caro *et al.*, 2017). Despite being present at multiple field sites (colonies were present at Plantel Matilde and it was seen during casual observations of non-buzz-pollinated plants here and at X'Kanchackan), *M. beecheii* was not recorded visiting any buzz-pollinated flowers. Why this species (which can buzz pollinate) would not visit flowers that it could effectively extract large amounts of pollen from, while most bee species visiting these flowers cannot, is unclear and somewhat puzzling. This could be a further demonstration of the high level of specialization seen in the pollination networks.

Pollen thieves accounted for over 75% of recorded floral visits. The activity of pollen thieves directly reduces plant reproductive fitness through competition with effective pollinators, and less efficient pollination services (Renner, 1983; Hargreaves *et al.*, 2009; Rego *et al.*, 2018; Page & Williams, 2023). Damage inflicted on flowers by thieves makes them less attractive to future potential visitors (Mescher *et al.*, 2020; Vega-Polanco *et al.*, 2020) and often makes buzz-pollinated flowers unusable by buzzing-bees (Mesquita-Neto *et al.*, 2021). This likely has a negative impact on plant reproductive success (Hargreaves *et al.*, 2009) and can even lead to population extinction (Rego *et al.*, 2018). Eusocial bees were by far the most abundant pollen thieves of buzz-pollinated plants in this study. Wild populations of *Trigona fulviventris*, *Nannotrigona perilampoides*, *Frieseomelitta nigra*, and *Cephalotrigona zexmeniae* naturally occur in the Yucatán, but these species are also managed in apiculture alongside the European honeybee (*Apis mellifera*) (Bratman, 2020; Brown *et al.*, 2020). Together these eusocial bees accounted for 28% of floral visits. Yet, native beekeeping has seen a resurgence in recent years (Bratman, 2020) and may result in greater population sizes than would occur naturally. We propose that, when their populations are artificially elevated due to apiculture, endemic eusocial bees likely have a strong impact on the structural organization and properties of

pollination networks and upon plant reproductive success, as reported for *Apis* (Dáttilo *et al.*, 2022), *Trigona* (Rego *et al.*, 2018) and *Bombus* bees (Dafni *et al.*, 2010). The impact of managed pollinators, and their successful conservation efforts, on the reproductive success of buzz-pollinated plants and their pollinators could be an interesting line for future research.

Conclusions

Field observations of buzz-pollinated plants provide a powerful mechanism to determine the impact of effective and ineffective pollinators on pollination networks as buzzing bees are audibly identified. Our work shows that plant-pollinator networks vary significantly when comparing legitimate pollinators against all floral visitors. We should look further into how pollination networks restructure considering this difference across other plant-floral visitor networks because this might change how we weigh the value of pollinators in a community, influencing conservation efforts and our valuation of managed and wild pollinator communities for crop production. As this work addressed networks from the pollinators perspective, future work should address important plant factors such as pollinator fit and herkogamy (Koski *et al.*, 2018; Mesquita-Neto *et al.*, 2021), weighting networks by pollen transfer efficiency and floral reproductive success.

Acknowledgements

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Chapter Six. General Discussion

My PhD investigated the chemical and behavioural ecology of buzz pollination across a range of scales from within-flower spatio-temporal scent variation, the use of scent variation by pollen foraging bees, and the ecological impact of legitimate and illegitimate floral visitors in natural communities of buzz-pollinated flowers. I will briefly describe the main results from each of my four data chapters below, and then proceed to discuss these findings in a broader context focused on (a) spatio-temporal scent variation, (b) pollen foraging behaviour, and (c) the implications these may have for the ecology of buzz-pollinated plants.

Chapter Two. Pollinator Responses to the Scent of Buzz-Pollinated Flowers with Morphologically Distinct Anthers

In Chapter Two I described the colour spectrum and olfactory signals produced by different organs of a buzz-pollinated plant with heterantherous flowers (*Solanum rostratum*) and assayed the impact of scent signals on a pollinator (*Bombus terrestris*). To my knowledge, this is the first study to explore the role of scent in regulating pollinator attraction and behaviour that investigates the combined aspects of buzz-pollination and heteranthy, despite the regular association between the two traits (Vallejo-Marín *et al.*, 2009; Barrett, 2021). Based on previous studies, I expected that bees would be more attracted to scent extracted from the androecium than the corollas (Solís-Montero *et al.*, 2018), and that floral vibrations would be triggered by scent extracted from the androecium (Russell *et al.*, 2018). Furthermore, I hypothesised that the division of labour between anther morphs, extensively explored from visual and morphological perspectives (Vallejo-Marín *et al.*, 2014; Barrett, 2021), would extend to scent and so influence pollinators. I therefore predicted that scents from feeding anthers would elicit greater visitation and more floral buzzing than those of pollinating anthers. These predictions were only partially realised. Bees were more attracted by the scent of the androecium, foraging for longer and producing more floral buzzes, compared to corolla scent. However, bee attraction and foraging behaviour did not differ between the scents of the two anther morphs despite differences in scent emission and composition. This shows that the morphological and visual differences between polymorphic anthers are not reflected in all channels of plant-pollinator signalling, possibly due to other pressures (i.e., florivory: Basso-Alves *et al.*, 2022).

Chapter Three: Pollen Removal Changes Floral Scent of Buzz-Pollinated *Solanum* Flowers

In Chapter Three I investigated a mechanism through which flowers with concealed rewards, such as pollen in buzz-pollinated plants, could provide pollinators with honest signals of reward availability: rapid temporal scent change. Honest signalling, whereby signals fluctuate with reward availability or quality (Wright & Schiestl, 2009), is hypothesised to benefit plants if it increases pollen import and export, and to benefit pollinators if it improves learning, handling and profitability (Dobson *et al.*, 2000; Raguso, 2004b). This has been explored when floral visitors can directly assess rewards (Burdon *et al.*, 2015, 2020), but it has not been studied where rewards are concealed as is the case with pollen in buzz-pollinated plants. By analysing the VOCs emitted by buzz-pollinated flowers with and without pollen, I identified changes in the amount and composition of floral scent emission. Linalool, a broad-spectrum pollinator attractant (Raguso & Pichersky, 1999), was only emitted by flowers containing pollen. In *Solanum lumholtzianum*, pollen removal correlated with significant decreases of linalool and farnesol, as well as overall scent emission. Although we did not identify the mechanism by which scent change occurred, bees are likely to be able to make use of this signal change as they have an acute sense of smell (Ramirez *et al.*, 2023) and can learn a diverse array of cues to make accurate foraging decisions (Raguso, 2004a; Clarke *et al.*, 2013; Looy *et al.*, 2014).

Chapter Four: Use of Scent Signals by Pollen Foraging Bees

Having demonstrated the spatial and temporal variation of floral scent that occurs in nectarless plants in Chapters Two and Three, I explored how pollen foraging bees use scent to inform foraging decisions in Chapter Four. Nectar has been the reward of choice in bee research since at least the 1950's (von Frisch, 1954). In contrast, pollen foraging has received little attention despite its crucial importance as both a source of complex nutrients for floral visitors (Nicholls & Hempel de Ibarra, 2017) and as the male gametophyte of all angiosperms (Dajoz *et al.*, 1991; Gurevitch *et al.*, 2006). As expected, bees initially chose to visit scented flowers, but bees learned to alter this preference if scent was not paired with reward presence. We confirmed previous findings that floral buzzing is stimulated by pollen-like substances and scent (Russell *et al.*, 2017, 2018) (Chapter Two). My study was, however, the first demonstration of a single scent compound (i.e., linalool) triggering flower-buzzing. Curiously, presenting scent and pollen-like rewards together stimulated less floral buzzing than either stimulus alone.

Chapter Five: Pollination Networks of Plants with Buzz-Pollinated Flowers in a Tropical Dry Forest Community (Yucatán, Mexico)

In my final data chapter, I investigated the natural pollination networks of buzz-pollinated plants and explored how network structures differ when distinguishing between floral visitors which are potential mutualists or pollen thieves (i.e., those which do and do not produce floral buzzes when

visiting buzz-pollinated plants, respectively) (Mesquita-Neto *et al.*, 2018). I found buzz-pollinated plants were regularly visited by pollen thieves, in some cases making the majority of visits. However, I found networks to be highly specialised with plant species sharing few pollinators, which may explain the lack of behaviour switching by bee species visiting different plants. I found sub-networks consisting of only buzzing bees to be more generalised than the overall visitation network.

Spatiotemporal Scent Signal Variation

The spatio-temporal variation of scent in buzz-pollinated flowers and its integral role in regulating pollinator attraction and behaviour was demonstrated in this thesis. Scent production of the buzz-pollinated heterantherous flower, *Solanum rostratum*, showed strong spatial partitioning between floral organs (Chapter Two). Here, I showed that the androecium played the predominant role in olfactory signalling, producing the most voluminous and chemically diverse scent, which proved most attractive to pollinators and triggered most floral buzzing. Temporal variation of floral scent emission was demonstrated in a clade of pollen-only buzz-pollinated plants which includes *Solanum rostratum*, whereby scent emissions fluctuated with pollen availability. I explored the hypothesis that such spatial and temporal scent variation has a role in plant-pollinator communication in behavioural experiments with bumblebees in Chapter Two and Chapter Four, demonstrating that scent does indeed influence pollen foraging in bees. This allows us to have some confidence that the variation in scent spatially across floral organs (Chapter Two) and temporally with pollen removal (Chapter Three) play functional roles in the behaviour of pollen foraging bees and so are likely plant adaptations.

Temporal Variation and Honest Signalling

Signals which correlate with reward quality or quantity can be viewed as honest signals as the information they provide is reliable (Raguso, 2004b; Wright & Schiestl, 2009). I showed that scent fluctuates with reward availability in Chapter Three and that bees use scent to select rewarding flowers in Chapter Four. Previous investigations which have described correlations between floral traits and reward have investigated nectar (Knauer & Schiestl, 2015; Gervasi & Schiestl, 2017; Parachnowitsch *et al.*, 2019). However, the difficulty encountered when investigating honest signals associated with nectar (Burdon *et al.*, 2020) may be because the flowers also produce pollen and so signals may be conflated. Pollen-only flowers may be ideal for exploring honest plant-pollinator communication as signals can only be related to pollen. Yet, to my knowledge, this was the first time where honest signals were explored in pollen-only flowers. Chapter Three also likely demonstrates the first report of indirect honest signalling, whereby the signal is produced by tissues other than the

reward. Further investigation is needed to test my Scented Pollen Hypothesis: that pollen produces scent, so pollen removal from flowers could change scent emission. If this hypothesis were supported, it would be necessary to determine whether scent can be effectively transmitted from pollen concealed within poricidal anthers, in which is concealed, to the environment.

I found that bees use scent to make pollen foraging decisions (Chapter Four), giving me confidence to assert that these dynamic signals allow bees to make accurate decisions when foraging on flowers with concealed floral rewards. This also provides a mechanism for honest signalling in flowers which are regularly emptied of rewards. For example, plants which employ reward dosing strategies, such as sequential pollen maturation (Dellinger *et al.*, 2019; Telles *et al.*, 2020; Kemp & Vallejo-Marín, 2021) or slow nectar replenishment (Raguso, 2004b; Wignall *et al.*, 2020), may be emptied of rewards by a single forager. Bees avoid uncertainty when foraging (Leonard *et al.*, 2011a; Austin *et al.*, 2019) so flowers which signal honestly are more likely to retain pollinators over those which do not. So, dynamic adjustment of floral signalling, as floral scent allows, is likely to be widespread and likely to be identified in many other pollination systems that rely on repeated visitation to maximise reproductive success.

Honest signalling is expected to evolve and persist where it proves beneficial, for example by increasing plant export and receipt of pollen (Raguso, 2004b; Sun *et al.*, 2018). However, the degree of pollinator dependence varies in buzz-pollinated flowers, with repeated transitions from pollinator-dependant to predominantly self-fertilizing strategies (Whalen, 1978b; Vallejo-Marín *et al.*, 2014). So, we would not expect all flowers to signal honestly, rather those which rely most heavily on pollinators and whose pollinators are scent-oriented, such as bees (Ramirez *et al.*, 2023). Traits such as heteranthery and herkogamy are hypothesised to be associated with pollinator dependence (Vallejo-Marín *et al.*, 2014; Solís-Montero & Vallejo-Marín, 2017). This likely explains why the larger flowers with more pronounced heteranthery and herkogamy (i.e., spatial separation of anthers and stigmas) in *Solanum* section *Androceras* used in Chapter Two (Vallejo-Marín *et al.*, 2014) produced signals which were associated with rewards. Conversely, the smaller, less heterantherous and herkogamous flowers did not show this signal variation. Bees are highly dependent on scent (Ramirez *et al.*, 2023) and can effectively learn associations between rewards and any perceptible signal (Kunze & Gumbert, 2001; Rains *et al.*, 2008; Clarke *et al.*, 2013; Pahl *et al.*, 2013; Harrap *et al.*, 2020). These, combined with my finding that bees will use scent to find pollen (Chapter Four and Chapter Two), provide compelling evidence that the more pollinator-dependent a plant, the more likely it is to produce honest signals.

In Chapter Three I described how vibrational pollen removal causes changes in the scent emissions of buzz-pollinated flowers (significant in *Solanum lumholtzianum* and detectable in *S. citrullifolium* and *S. grayi* ssp. *grandiflorum*), then proposed and discussed two potential mechanisms for this change. The Scented Pollen Hypothesis states that pollen is a source of VOC, the removal of which changes floral scent. The Damage Hypothesis proposes that damage incurred by flowers during buzz pollination precipitates changes in floral scent. Here I propose a third, more controversial explanation: the Detection Hypothesis. Plants reportedly respond to vibrations in contexts of prey capture in the Venus flytrap (*Dionaea muscipula*) (Hedrich & Neher, 2018), herbivore detection (Peiffer *et al.*, 2009; Appel & Cocroft, 2014; Velilla *et al.*, 2020) and water seeking in roots (Gagliano *et al.*, 2012). The vibrations experienced by flowers during buzz-pollination are greater than are required to initiate changes in *Arabidopsis thaliana* metabolism in response to invertebrate herbivory (Appel & Cocroft, 2014; Pritchard & Vallejo-Marín, 2020), so may be detectable by the plant and cause changes in floral scent emission. However, further investigation is needed to disentangle which mechanism causes scent changes found in Chapter Three. Regardless of the mechanism, however, the wealth of research into cues bees can learn to make foraging decisions (Kunze & Gumbert, 2001; Rains *et al.*, 2008; Clarke *et al.*, 2013; Pahl *et al.*, 2013; Harrap *et al.*, 2020) makes it likely that bees could use these changes to make foraging decisions.

Spatial Variation and Signal Coordination

Significant spatial variation of floral visual and olfactory signals was shown in Chapter Two. Coordination of these signals likely plays a role in pollinator attraction and positioning. Corollas emitted little scent but were large and visually attractive with significant ultraviolet reflectance, probably playing a role in long-distance attraction, as in *Solanum houstonii* (Russell *et al.*, 2018). In contrast, the androecium is UV absorbent (Figure 12, Figure 13) and strongly scented (Figure 6). This is highly reminiscent of the general bull's eye pattern found in many bee-pollinated flowers (Tunes *et al.*, 2021; Lunau *et al.*, 2021; Basso-Alves *et al.*, 2022). Coordination of visual and olfactory signals could function across different spatial scales. Visually, corollas provide a long-range signal (Russell *et al.*, 2018) and the smaller, UV absorbent androecium may provide contrast to help position bees within the flower (Figure 13) and may also act as a pollen mimic (Lunau & Wester, 2017; Lunau *et al.*, 2020). An airborne concentration gradient of scent also originates in the floral centre due to the scent-emitting androecium (Chapter Two) (Solís-Montero *et al.*, 2018). Pollen foragers attracted to these flowers could use honest scent variations described above (Chapter Three) to decide whether to land or not (Chapter Two, Chapter Four). Finally, once landed, scent would trigger floral vibration production (Chapter Two and Chapter Four) (Russell *et al.*, 2018). The off-centre positioning of pollinating anthers in heterantherous plants, such as *Solanum rostratum* (Figure 2, Figure 5) (Vallejo-

Marín *et al.*, 2014; Dellinger *et al.*, 2019; Barrett, 2021), and different visual and olfactory properties described in Chapter Two, likely means they are rarely gathered by bees and buzzed directly. So, it is likely that the close grouping of anthers and floral structural properties are critical in vibration transmission to pollinating anthers (Nunes *et al.*, 2021; Nevard *et al.*, 2021) and subsequent pollen deposition on “safe sites” of the bees’ body (Figure 2) (Koch *et al.*, 2017).

The specificity and coordination of signals demonstrated here may be particularly important for plants with specialised flower morphologies, such as those associated with buzz-pollination (Harder & Barclay, 1994; De Luca & Vallejo-Marín, 2013). As reward collection requires specific pollinator positioning and behaviour, if pollinators are attracted but cannot collect rewards, they are unlikely to visit conspecific plants. Precise bee positioning is necessary in buzz-pollinated plants, so it is likely that the signals act to orient pollinators. What remains to be explored is the impact of flower architecture on pollinator positioning: epidermal cells are specialised in many flower types affecting morphological, attractive, and structural traits (Glover *et al.*, 2004; Whitney *et al.*, 2009b,a; Falcão & Stehmann, 2018; Garcia *et al.*, 2020). We would expect buzz-pollinated flowers to benefit especially from flower epidermis specialisation which effects bees’ grip (Whitney *et al.*, 2009a) and so pollinator positioning and foraging efficiency. Reflexed corollas are also common in buzz-pollinated flowers, especially those with the typified *Solanum*-type flower (Faegri, 1986; Corbet *et al.*, 1988; De Luca & Vallejo-Marín, 2013). Similarly, many buzz-pollinated species produce pendant-oriented flowers (e.g., *Echeandia luteola*: Figure 24, Appendix 18) which influences outcrossing (Nevard & Vallejo-Marín, 2022). How reflexed corollas and pendulous flowers influence vibration transmission through pollinator orientation and attachment, and airborne pollen-streams are interesting questions which remain to be explored and contrasted with other buzz-pollination adaptations such as pollen ricocheting in *Chamaecrista* (Amorim *et al.*, 2017, 2019).

Evolution of Heteranthery

The Division of Labour Hypothesis of heteranthery states that anther form is specialized to the divergent functions of pollinator reward and plant fertilization (Vallejo-Marín *et al.*, 2009; Dellinger *et al.*, 2019; Telles *et al.*, 2020; Kay *et al.*, 2020). I therefore expected the visual and morphological specialisation of anthers in heterantherous plants to extend to olfactory signals. In *Solanum rostratum* this was not so (Chapter Two): the olfactory signals of feeding and pollinating anthers were both able to attract bees and stimulate buzzing. Heteranthery evolves due to other pressures too. In the case of *Clarkia* (Onagraceae), heteranthery provides a pollen dosing mechanism, allowing pollen to be presented over a longer time period (Peach & Mazer, 2019). This increases male fitness and saves energetic investment by extending the time a flower performs its male function effectively (Peach & Mazer, 2019; Kay *et al.*, 2020).

Plant reproductive fitness is impacted by herbivory (Theis *et al.*, 2007; Dicke, 2009; Scala *et al.*, 2013; Higuchi & Kawakita, 2019). In *Solanum rostratum* herbivores more regularly consume feeding anthers than pollinating anthers without reducing female reproductive success (i.e., fruit and seed set) (Vega-Polanco *et al.*, 2020). This herbivore preference may be due to the conspicuousness of feeding anthers due to their pollinator-attractive signals (Chapter Two) (Mesquita-Neto *et al.*, 2017; Solís-Montero *et al.*, 2018; Velloso *et al.*, 2018; Valadão-Mendes *et al.*, 2022; Basso-Alves *et al.*, 2022). However, pollinating anthers often remain in the flower after florivory (Basso-Alves *et al.*, 2022). Thus, the capacity to attract pollinators and stimulate floral buzzes (Chapter Two, Chapter Four) (Russell *et al.*, 2018) in the absence of feeding anthers could ensure male reproductive success after florivory. This leads me to propose an alternative hypothesis for the evolution of heteranthery: the Herbivory Evasion Hypothesis. Here, centrally placed, conspicuous anthers are consumed by florivores while cryptic, decentralised anthers are overlooked. Therefore, flowers retain the capacity to attract and position pollinators, securing pollen deposition and female fitness. This would result in the evolution of flowers with greater degrees of anther polymorphism. Furthermore, pollen contained within the remaining anthers may still be released and reward pollinators or fertilize ovules. Fittingly, under this hypothesis feeding and pollinating anther morphs would retain these names according to their function.

The Herbivory Evasion Hypothesis may also help explain the regular association of heteranthery with buzz-pollinated and pollen-only flowers. Buzz-pollinated plants may represent a high-quality food source for non-pollinating pollinivores, because the reduced number of pollinator clades (Buchmann *et al.*, 1983; Vallejo-Marín & Vallejo, 2021; Nevard *et al.*, 2021) and pollen dosing strategies (Harder & Thomson, 1989; Harder & Wilson, 1994; King & Buchmann, 1996; LeBuhn & Holsinger, 1998; Kemp & Vallejo-Marín, 2021) means they are likely to contain more pollen at any given time. In pollen-only flowers pollen consumption represents both the loss of male gametes and of the only reward to secure pollinator visitation. It can therefore also impede female function, effectively neutering the flower. In such situations the morphological adaptations of pollinating anthers, their off-centre position (Figure 2)(Papaj *et al.*, 2017; Velloso *et al.*, 2018; Barrett, 2021), and their different visual and olfactory properties (Chapter Two) (Solís-Montero *et al.*, 2018; Basso-Alves *et al.*, 2022), likely means they are consumed more rarely (Vega-Polanco *et al.*, 2020) retaining some reproductive capacity. Further quantitative studies will be critical to a complete understanding of scent use by heterantherous species.

Overall, these results suggest that the spatial and temporal variation of scent signals play a vital role in regulating pollinator attraction, orientation, and behaviour during a floral visit. Coordination of signals at distance attracts pollinators, honest temporal scent variation informs pollinator visitations,

spatial scent variation regulates positioning of pollinators on the flower, and scent triggers bee buzzing behaviour with implications for effective functioning of separate anther morphs in heterantherous flowers. This level of control may be especially necessary in plants where pollination success is predicated on both the position and behaviour of a visiting bee, as in buzz-pollinated plants. Although a large body of work has been undertaken to understand bees' visual and olfactory systems, we still do not fully understand how pollinators address the complementary or redundant nature of signals conveying the same information (Rypstra *et al.*, 2009; Akçay & Beecher, 2019). Thus, this system could be ideal for the study of the evolution, maintenance, and loss of complex traits.

Use of Scent Signals when Pollen Foraging

As noted above, I found strong evidence for the spatial and temporal variation of scent in pollen-only flowers (Chapter Two and Chapter Three, respectively). However, whether bees use scent when foraging for pollen remained an open question which needed addressing to demonstrate that these fluctuations could play a role in plant-pollinator communication. Compared to nectar foraging, little is known about pollen foraging (Nicholls & Hempel de Ibarra, 2013, 2017; Nicholls & De Ibarra, 2014; Muth *et al.*, 2015, 2016b; Russell *et al.*, 2016a, 2017). So, I asked: do pollen foraging bees use scent to inform decisions when foraging for visually concealed pollen? This question is pertinent as, if bees do not use scent when pollen foraging, then the spatio-temporal variation in scent emission shown in Chapter Two and Chapter Three are unlikely to be related to plant-pollinator signalling, but rather to other biological or ecological factors such as herbivory, florivory, time of day, or something else entirely. My results from both Chapter Two and Chapter Four confirm that bees use scent when pollen foraging: bees use scent to select flowers, but also learn to modulate their response to scent if it proves unrewarding. However, our research found some peculiarities too.

Research has shown that bee learning often occurs when flowers are unequal in some reward-associated characteristic: reward presence (Chapter Four), toxicity, bitterness, quality, concentration (Tiedeken *et al.*, 2014; Francis *et al.*, 2016; Muth *et al.*, 2016a; Koch & Stevenson, 2017; Rivest & Forrest, 2019; Austin *et al.*, 2019; Jacquemart *et al.*, 2019). However, all artificial flowers of Chapter Two were equally rewarding which begs the question: why did bees develop scent preferences? Though rewards were accessible to scrabbling behaviour (i.e., knocking or shaking loose pollen with legs or mandibles; Russell *et al.*, 2017), they were more readily gathered by floral buzzes. So, bees likely perceive treatments which elicit more buzzes (i.e., all androecium treatments) as more rewarding. This may explain bees developing a preference for androecium treatments compared to the corolla and control treatments. However, preferences did not develop between different anther

treatments. Rather, it appeared that learning occurred when making a mistake was riskier, namely, when the chance of choosing perceptively unrewarding flowers was greater than the chance of encountering rewarding ones. This difference in reward perception between researchers and their study organisms must be accounted for when designing experiments.

My results show that bees can detect pollen when foraging, but that pollen assessment is not immediate (Chapter Four). Indeed, preferences developed between foraging bouts in Chapter Two which suggests that some bee learning occurs in the colony as in Molet *et al.* (2009), perhaps by social learning (Bridges *et al.*, 2023). The slower rate of learning with pollen, compared to nectar, may account for the abundance of pollen mimicking systems (Lunau, 2000, 2006) but scarcity of nectar mimicry (Lunau *et al.*, 2020). Furthermore, if bees learn to associate pollen with cues slowly it may help explain why bees ability to assess pollen quality and quantity while foraging is still contested (reviewed in Nicholls & Hempel de Ibarra, 2017), as many experiments do not allow bees a memory consolidation period (e.g., Ruedenauer *et al.*, 2015; Muth *et al.*, 2016; Russell & Papaj, 2016). I feel that this aspect of memory formation and learning in bees requires future investigation (e.g., Konzmann & Lunau, 2014; Nicholls & Hempel de Ibarra, 2017). Incorporating different types of foraging behaviour, flower complexity and reward immediacy could provide insights into pollinator learning, behaviour and thus pressures driving floral evolution.

Stimulation of Floral Buzzing

Flower buzzes can be triggered by VOC. Results from Chapter Two confirmed prior findings that floral scent triggers floral buzzing by bees (Russell *et al.*, 2018). To my knowledge, however, Chapter Four is the first demonstration of a single VOC triggering this complex behaviour, in this case linalool. In Chapter Three, linalool was one of two VOCs identified as potentially mediating honest signals. As the relative abundance of chemicals in scent-blends can be used by bees to make foraging decisions (Lawson *et al.*, 2018), linalool potentially has a role in triggering buzzing. Yet, the presence of linalool in many flower families which are not buzz-pollinated means it is very unlikely to be the sole olfactory trigger. Further research is needed, including electroantennographic studies, to identify the chemical triggers for buzz pollination. Furthermore, the fact that linalool was detected in the scent of *S. rostratum* in Chapter Two but not in Chapter Three, suggests that extraction methods (hexane solvent and dynamic headspace, respectively) vary in their efficacy. This emphasises the need to use multiple collection techniques and methodological standardization when assessing VOCs to ensure every scent component is detected.

Bees buzzed when visiting unscented flowers which contained pollen-like rewards (Chapter Two and Chapter Four) as found by Russell *et al.* (2018). However, they also buzzed scented flowers which

were unrewarding and even flowers which were unrewarding and unscented in Chapter Four. Touch undoubtedly plays a role in pollen foraging and, without other sensing mechanisms, may explain observations of bees collecting inert granular pollen-like substances (i.e., glass beads, plastic granules, and cellulose powder) (Arenas & Farina, 2012; Konzmann & Lunau, 2014). However, unwanted pollen is regularly groomed from the body and discarded (Azo'o *et al.*, 2011; Lunau *et al.*, 2015), suggesting quality assessment occurs post-collection. Detection could be through tactile cues, chemoreceptors (Nicholls & Hempel de Ibarra, 2013; Pearce *et al.*, 2017), taste (Michener *et al.*, 1978; Michener, 2007b; Raine & Chittka, 2007a), or nutritional assessment via proxies such as pollen surface proteins or sugars (Leonhardt & Blüthgen, 2012; Ruedenauer *et al.*, 2019). Indeed, why bees buzzed less when reward and scent were presented together, compared to when presented separately, (Chapter Four) needs further exploration. I suggest an explicit, detailed exploration into how olfactory and tactile cues influence buzzing. Likewise, other tactile cues may also play a role in triggering buzzing in bees which deserve exploration: how does the shape of buzz-pollinated flowers influence bee positioning and behaviour, especially the convergently evolved *Solanum*-type flower (Vogel, 1978; Faegri, 1986; De Luca & Vallejo-Marín, 2013)?

The experimental exploration of pollen foraging proved especially challenging during this PhD, so I hope readers will take advantage of the developments and protocols presented in this thesis when designing future research. As pollen foraging is needed for the reproductive health of almost 90% of flowering plants, including for 35% of the food we eat, it has global implications (Ollerton *et al.*, 2011; Potts *et al.*, 2016; Rodger *et al.*, 2021), especially in the face of global ecological decline (Biesmeijer *et al.*, 2006; Straka & Starzomski, 2014; Zattara & Aizen, 2021). Without a detailed understanding of pollen ecology, how can we possibly predict the impacts our actions will have?

Ecological Implications of Floral Scent

My research on buzz-pollination presents wider implications for pollination ecology. Chapter Five revealed that buzz-pollinated plants often receive many visits from pollen thieves. It may be that plants with more generalist pollination strategies also receive a similar number of visits from poor pollinators. However, as they lack a distinctive pollination mode, all floral visitors are considered pollinators. This fieldwork also revealed high variation in the number and proportion of floral visits by legitimate pollinators across different geographical locations. However, a major limitation of this fieldwork was the short survey period, which likely provided an incomplete picture of pollinator communities, not least because populations fluctuate both within (Carril *et al.*, 2018) and between years (Williams *et al.*, 2001; Wilson *et al.*, 2009). Furthermore, ecological communities consist of a few common species and many rare species (Winfree *et al.*, 2015; Russo *et al.*, 2015); the rare

species are most likely to be detected during longer research periods. Despite this, the abundance of non-buzzing bees, especially managed species that comprised almost 1/3 of all floral visitors, raises real questions about the susceptibility of these plants to changing pollinator communities.

The complexity and specialization of the buzz-pollination system, as demonstrated in Chapter Two, Chapter Three and Chapter Four, may make it especially susceptible to other man-made perturbations such as pesticides, pollution, climate change and associated phenological shifts (Straka & Starzomski, 2014; Whitehorn *et al.*, 2017; Kenna *et al.*, 2019; Carter *et al.*, 2020; Gérard *et al.*, 2020; Warren *et al.*, 2021; Jaworski *et al.*, 2022; EASAC, 2023). This could have a profound cascade of impacts on ecosystems due to the abundance of buzz-pollinated plants (Buchmann *et al.*, 1983), and human agriculture due to the importance of certain buzz-pollinated crops (Cooley & Vallejo-Marín, 2021). Although there is scant evidence about the impact of pesticides on pollen foraging (Whitehorn *et al.*, 2017), its' wider impacts are well known (EASAC, 2023). Greater understanding of this topic has implications for pollinator health. For example, human activities which impair pollinator cognition, such as pollution and the use of pesticides (McFrederick *et al.*, 2009; Muth *et al.*, 2019; Cullen *et al.*, 2019)) would be expected to cause bees to make poorer foraging decisions, thus reducing fitness and increasing local extinction risk. This might be especially problematic for specialised pollination systems such as buzz-pollination. Furthermore, we are starting to realise the impacts of pesticides on multi-trophic plant interactions. For example, herbicides (glyphosate) reduce plants' ability to form relationships with symbiotic microbes with cascading effects on plant-pest insect interactions (Fuchs *et al.*, 2023). As we know about similar cascades which impact plant-pollinator interactions, I expect it is only a matter of time until similar impacts on plant-pollinator interactions are also revealed.

Concluding Remarks

Scent is a key component of the conspicuous displays used by plants to communicate with their pollinators. Yet we are only beginning to understand the complex ways in which scent regulates plant-pollinator interactions. Buzz-pollinated plants have specialised flowers which require specific positioning and behaviour from their pollinators. Spatio-temporal variation in scent is used to orient bees within buzz-pollinated flowers for effective pollen transfer, as well as triggering the production of vibrations necessary to release pollen. Future research should explore the underlying mechanisms which trigger floral buzzing in bees. This should provide insight into the evolution of both the buzz-pollinated flower and buzzing behaviour in bees. Furthermore, I predict that wider exploration of dynamic scent signalling and its role in plant-pollinator interactions will provide invaluable insight

into the biology and ecology of plant-pollinator interactions, especially from the perspective of honest floral signalling.

The very degree of specialisation which makes pollination research compelling, exemplified by control of pollinators visiting buzz-pollinated plants, may make it susceptible to perturbations. This is particularly relevant when considering the changes occurring globally to ecosystems due to human activity such as land-use change, agrochemical use, pollution, and climate change. These human impacts could conceivably lead to breakdowns in pollination mutualisms, reducing plant reproductive success, with widespread impacts on ecosystem services for ecology and agriculture.

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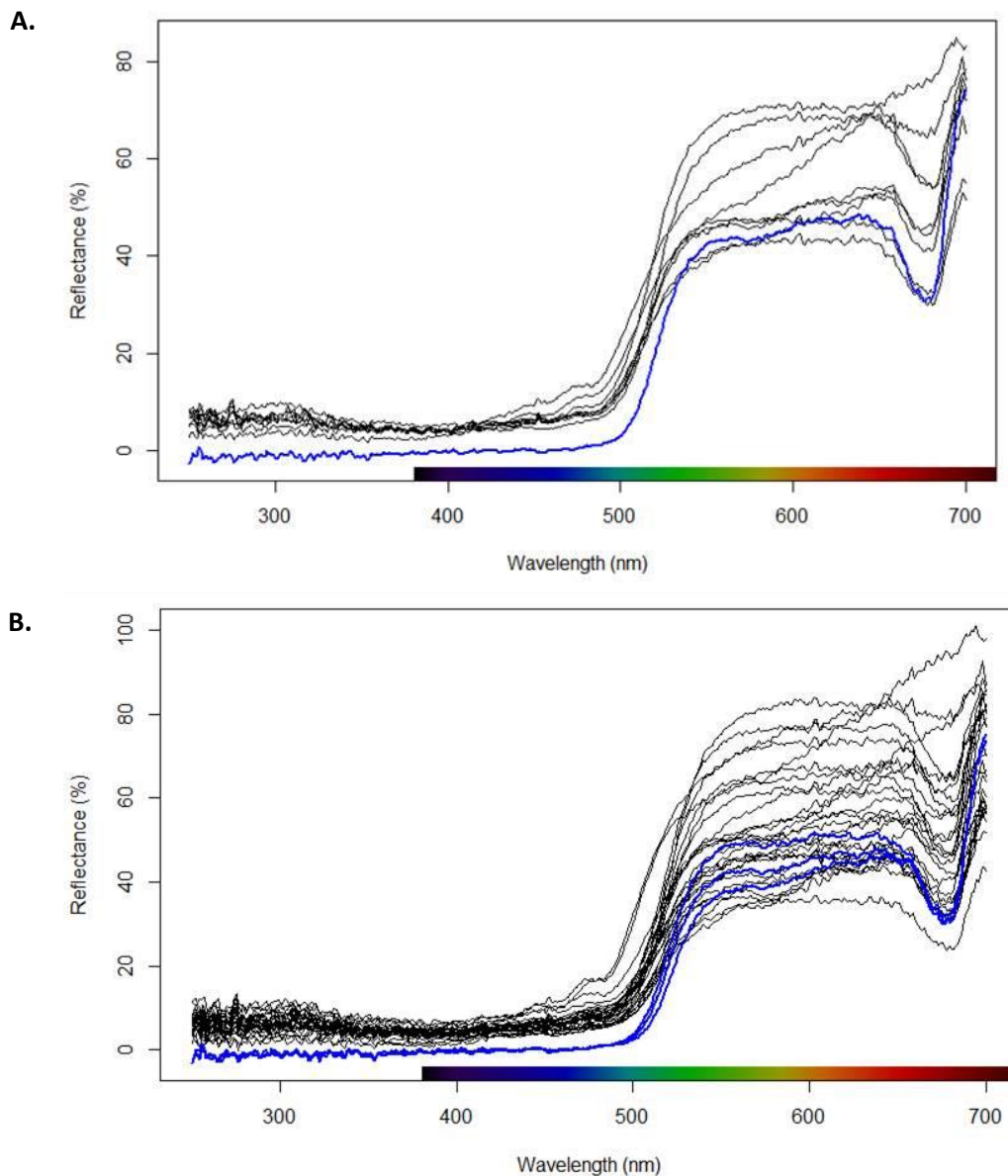
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Appendices

Appendix 1. Investigation of all pollinating anther base spectral readings highlighted an anomaly. All readings of one pollinating anther base (blue lines) contained high numbers of theoretically impossible negative reflectance readings below 465nm nm. This individual was therefore eliminated from analyses. Appendix 1A shows mean reflectance and Appendix 1B raw reflectance for each pollinating anther base observation.

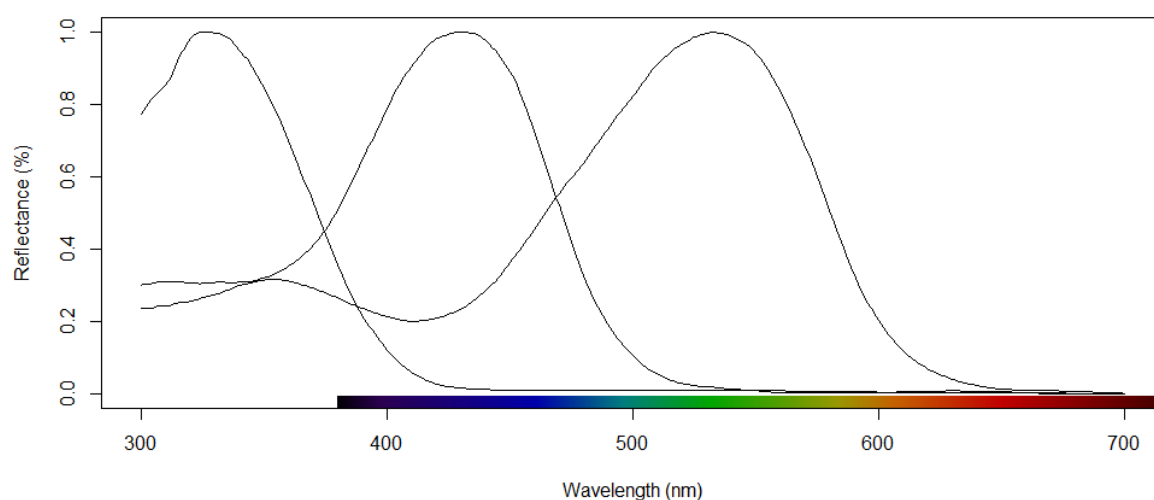


Appendices

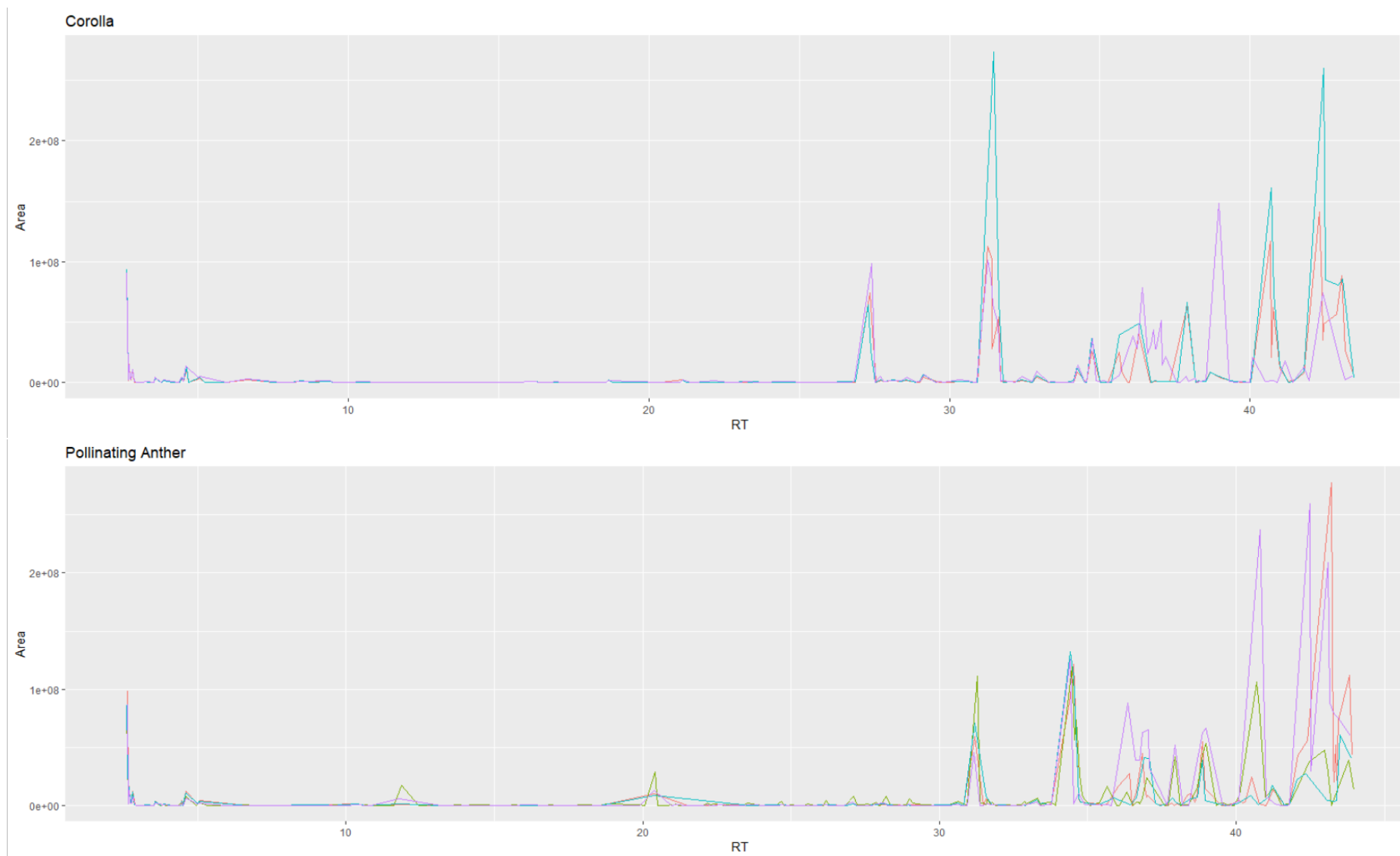
Appendix 2. Bee vision modelling suggests *Bombus terrestris* can discriminate between all floral organs of *Solanum rostratum* (mean hexagonal units >0.09, from (Dyer *et al.*, 2008)).

Organ 1	Organ 2	Hexagonal units (dS)		
		Mean	Lower	Upper
Pollinating anther tip	Corolla	2.52	0.35	5.27
Pollinating anther tip	Feeding Anther	2.29	0.42	5.02
Pollinating anther tip	Pollinating anther base	2.87	0.86	5.17
Corolla	Feeding Anther	0.36	0.21	3.82
Corolla	Pollinating anther base	0.40	0.16	3.59
Feeding Anther	Pollinating anther base	0.59	0.14	3.71

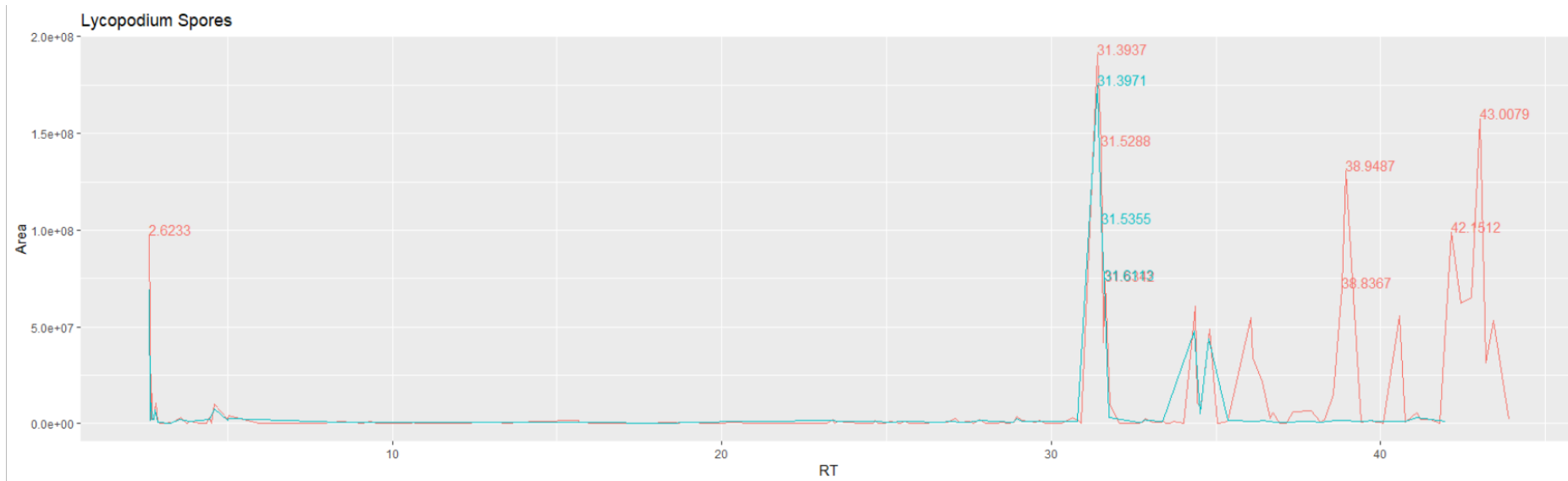
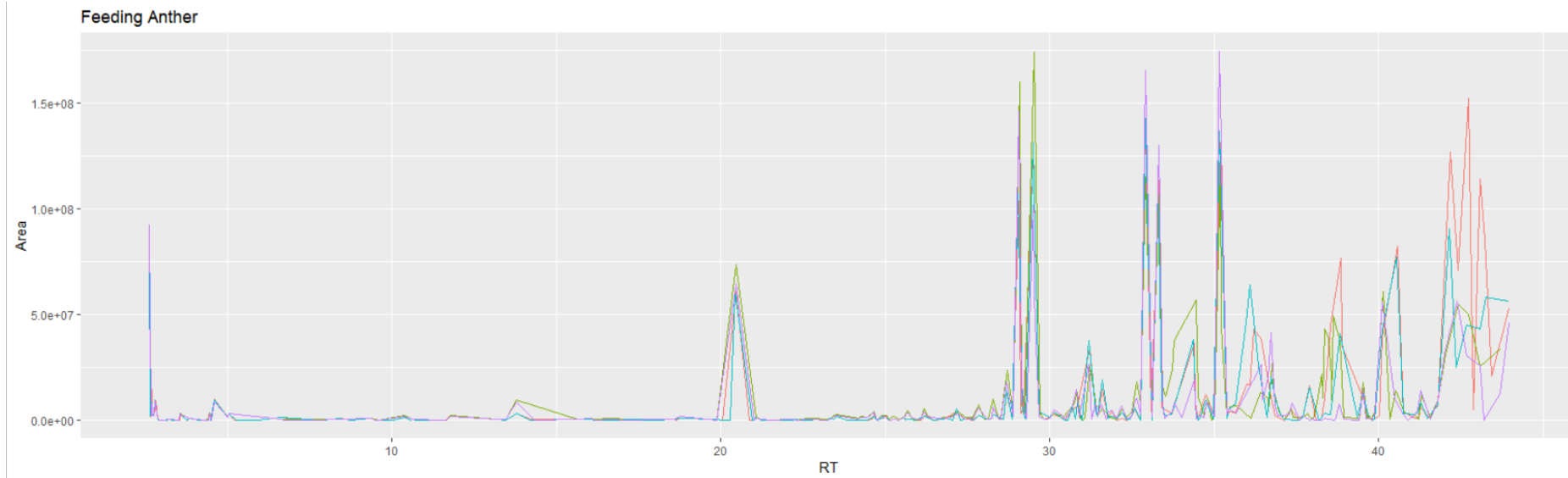
Appendix 3. Sensitivity of the trichromatic colour vision of *Bombus terrestris* photoreceptors to ultra-violet, blue and green, adapted from Peitsch *et al.* (1992). Colour scale-bar represents human vision.



Appendix 4. Spectrographs of GC-MS analyses of floral extracts.



Appendices



Appendices

Appendix 5. Mean emission of all volatiles detected in the headspace of *Solanum* section *Androceras*. Contaminant VOC eliminated from analyses (*) and SD.

	<i>S. citrullifolium</i>		<i>S. heterodoxum</i>		<i>S. rostratum</i>		<i>S. fructu-tecto</i>		<i>S. grayi ssp. grandiflorum</i>		<i>S. grayi ssp. grayi</i>		<i>S. lumholtzianum</i>	
Sample size (n)	4		4		3		4		4		4		4	
Condition	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen	+Pollen	-Pollen
Z3-Hexenyl Acetate	42.4	4.1	7.9	24.0	6.6	1.7	0.0	0.0	11.0	2.7	75.2	36.5	12.9	9.8
(ng minute ⁻¹ flower ⁻¹)	26.6	7.0	13.8	33.0	5.1	3.0	0.0	0.0	10.5	4.6	60.9	7.7	18.3	13.8
Z3-Hexenol*	14.0	18.3	30.6	17.3	12.0	11.7	144.5	60.5	17.1	9.4	21.6	36.0	88.8	61.0
(ng minute ⁻¹ flower ⁻¹)	6.9	11.2	8.7	3.1	11.6	0.7	119.9	14.9	10.9	7.0	7.9	16.5	59.0	28.0
Linalool (ng minute ⁻¹ flower ⁻¹)	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.0	498.1	0.0
	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.7	0.0	0.0	0.0	126.8	0.0
Caryophyllene	26.2	1.4	15.9	12.5	0.0	0.0	11.3	0.0	86.4	11.9	38.1	52.3	0.0	0.0
(ng minute ⁻¹ flower ⁻¹)	22.5	2.4	27.5	21.6	0.0	0.0	19.5	0.0	102.4	20.7	25.1	6.3	0.0	0.0
Methyl Phenylacetate*	51.6	3.9	62.9	27.2	21.2	13.3	50.1	43.9	13.8	8.9	21.6	54.2	164.1	33.4
(ng minute ⁻¹ flower ⁻¹)	30.5	4.0	57.1	31.8	27.6	16.4	21.4	21.1	10.2	8.0	24.1	47.4	35.9	16.3
Geraniol*	189.7	11.2	42.1	76.2	14.5	13.6	182.4	134.6	30.7	8.2	196.4	126.0	999.9	83.9
(ng minute ⁻¹ flower ⁻¹)	133.5	7.0	29.6	69.4	9.0	7.5	125.3	50.9	18.3	4.1	130.3	148.8	338.4	39.2
Butylated hydroxytoluene*	73.8	91.7	158.2	98.1	21.5	18.7	80.8	141.1	65.9	36.4	100.0	130.2	367.3	231.1
(ng minute ⁻¹ flower ⁻¹)	77.5	126.5	158.6	99.4	37.2	32.4	140.0	244.4	86.3	37.1	101.4	184.6	116.3	166.7
Farnesal	44.0	34.8	54.8	78.7	11.1	14.4	110.8	87.4	20.0	10.4	49.7	64.3	85.6	52.9
(ng minute ⁻¹ flower ⁻¹)	33.7	19.4	56.2	86.4	8.2	11.6	58.1	105.8	14.7	6.0	35.0	30.2	54.2	15.7
Farnesol	142.7	11.7	17.7	20.3	25.3	2.3	25.3	34.2	21.8	13.2	34.3	35.1	171.2	4.7
(ng minute ⁻¹ flower ⁻¹)	92.6	7.1	30.7	12.6	41.4	2.5	25.9	41.3	13.0	13.5	29.6	37.3	140.5	6.7
Total Emission	588.6	177.0	390.2	354.3	112.3	75.7	605.1	501.6	270.0	101.1	537.1	534.6	2388.0	476.8
(ng minute ⁻¹ flower ⁻¹)	347.9	159.4	283.9	242.0	64.5	35.1	218.8	348.7	249.7	24.9	308.7	235.2	314.9	172.8

Appendix 6. Retention times, Kovats Indices and molecular masses of compounds emitted by *Solanum rostratum* and their identification with FID and by Solís-Montero *et al.* (2018, * indicates identities confirmed by comparing against synthetic compounds). Retention times and Kovats Indices were established in this study for all volatiles below using synthetic chemicals († denotes those which were not).

Compound	Class (pubchem)	Retention time	Kovats Index	Molecular weight (g/mol)	Solis Montero <i>et al.</i> (2018)	This Study
Z3-Hexenyl Acetate	Carboxylic acid esters	5.990	1294	142.2		Yes
Z3-Hexenol	Fatty alcohol	6.877	1364	100.159		Yes
α-Copaene	Sesquiterpene	8.134	1469	204.357	Yes	
Linalool	Acyclic monoterpenoids	8.887	1533	154.25		Yes
Methyl benzoate / Benzoic acid methyl ester	Benzoic acid esters	9.689	1601	136.15	Yes*	
ethyl benzoate / Benzoic acid ethyl ester	Benzoic acid esters	10.183	1647	150.177	Yes*	
(E,E)-α-Farnesene	Sesquiterpenoids	11.041	1727	204.351	Yes	
γ-Decalactone	Gamma butyrolactones			170.25	Yes*	†
Hexadecane	Alkanes			226.44	Yes*	†
Methyl Phenylacetate	Benzene and substituted derivatives	11.205	1742	150.175		Yes
Methyl salicylate	Salicylates	11.374	1758	452.7	Yes*	
Ethyl salicylate	O-hydroxybenzoic acid esters			166.17	Yes*	†
Caryophyllene	Sesquiterpenoids	11.857	1803	204.357		Yes
Geraniol	Acyclic monoterpenoids	12.186	1837	154.253		Yes
Methyl eugenol	dimethoxybenzenes	13.834	2006	178.23	Yes	
Eugenol / Methoxy eugenol	methoxyphenols	15.169	2155	164.2	Yes*	
Farnesal isomer 1	Sesquiterpenoids	15.674	2213	220.35		Yes
Farnesal isomer 2	Sesquiterpenoids	15.774	2225	220.35		Yes
Farnesal isomer 3	Sesquiterpenoids	16.074	2261	220.35		Yes
(E,E)-Farnesol	Sesquiterpenoids	16.782	2347	222.37		Yes
Butylated hydroxytoluene	Phenylpropanes			220.356		Yes†

Appendices

Appendix 7. 2-Way ANOVA with Type III corrections was used to assess the impact of plant taxa and pollen presence on total floral VOC emission (Total Floral VOC Emission~ Pollen Presence*Plant Taxa).

	Sum Sq	DF	F-value	Relative P-Value
Intercept	269,028	1	24.2	1.5 ⁻⁰⁵ ***
Pollen Presence	86,022	1	7.7	0.0081 **
Plant Taxa	1,139,858	6	17.1	1.1 ⁻⁰⁹ ***
Pollen Presence: Plant Taxa	631,545	6	9.5	1.8e ⁻⁰⁶ ***
Residuals	444,474	40		

Appendix 8. Difference in VOC emission within taxa was tested with pairwise t-tests. Significant results are in bold.

Plant taxon	t	Df	p-value
<i>S. citrullifolium</i>	2.41	3.28	0.088
<i>S. heterodoxum</i>	-0.55	5.28	0.602
<i>S. rostratum</i>	0.94	3.26	0.412
<i>S. fructu-tecto</i>	0.27	4.33	0.797
<i>S. g. grandiflorum</i>	1.29	3.15	0.284
<i>S. g. grayi</i>	0.14	3.36	0.900
<i>S. lumholtzianum</i>	7.99	2.30	0.010

Appendix 9. MANOVA summary of the five VOC emitted by each taxa and the impact of pollen removal from flowers on their presence.

Summary	DF	Pillai	Approximate F	DF	Den DF	P
Pollen Presence	1	0.64805	13.2575	5	36	2.4 ⁻⁰⁷ ***
Taxon	6	1.88302	4.0274	30	200	1.4 ⁻⁰⁹ ***
Interaction	6	1.39277	2.5740	30	200	5.3 ⁻⁰⁵ ***
Residuals	40					

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

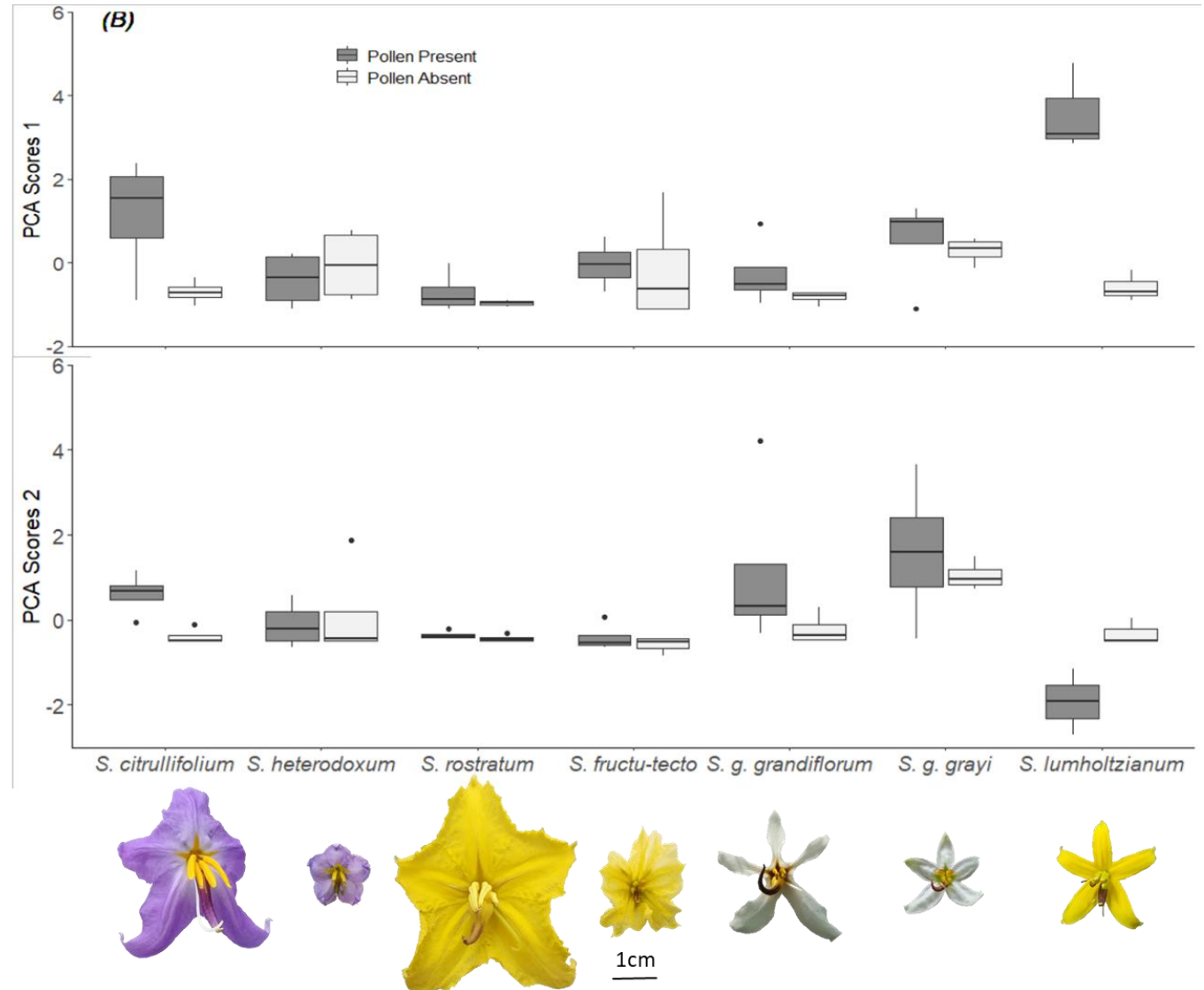
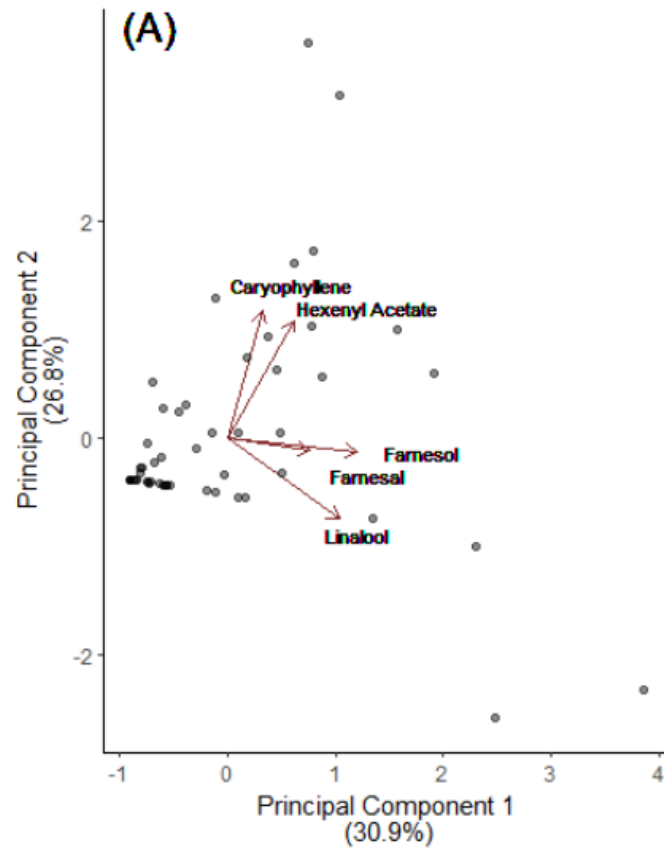
		DF	Sum Sq	Mean Sq	F-Value	P
Hexenyl acetate	Pollen Presence	1	1743.4	1743.39	2.7406	0.105654
	Taxon	6	17033.7	2838.94	4.4628	0.001514 **
	Interaction	6	4915.9	819.32	1.2880	0.284771
	Residuals	40	25445.4	636.13		
Linalool	Pollen Presence	1	42974	42974	35.455	5.478e-07 ***
	Taxon	6	329307	54885	45.282	2.613e-16 ***
	Interaction	6	329307	54885	45.282	2.613e-16 ***
	Residuals	40	48483	1212		
Caryophyllene	Pollen Presence	1	2950	2949.9	2.1527	0.15014
	Taxon	6	19648	3274.6	2.3897	0.04563 *
	Interaction	6	10051	1675.2	1.2225	0.31521
	Residuals	40	54813	1370.3		
Farnesal	Pollen Presence	1	185	185.1	0.0584	0.81021
	Taxon	6	45770	7628.3	2.4085	0.04419 *
	Interaction	6	4447	741.2	0.234	0.96289
	Residuals	40	126686	3167.2		
Farnesol	Pollen Presence	1	22469	22468.7	7.1819	0.01064 *
	Taxon	6	38828	6471.3	2.0685	0.07872 .
	Interaction	6	54816	9135.9	2.9202	0.01858 *
	Residuals	40	125141	3128.5		

Appendix 10. Component weightings of PC1 and PC2. The total variation explained by principal components one and two was 57.7% (30.9% and 26.8% respectively).

Floral Volatile	Component 1	Component 2
Hexenyl Acetate	0.403	0.708
Linalool	0.683	-0.494
Caryophyllene	0.210	0.762
Farnesal	0.502	-0.074
Farnesol	0.787	-0.090

Appendices

Appendix 11. Principal Component Analysis of VOC emission of *Solanum* section *Androceras*. **(A)** Biplot of PC1 and PC2 demonstrating the loadings of each of the six VOC, % variance explained by each PC presented in axis titles. **(B)** Principal Component 1, **(C)** Principal Component 2.

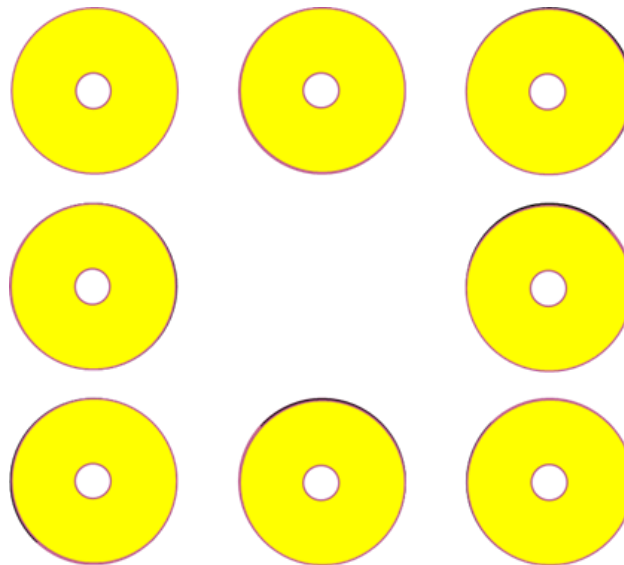


Appendix 12. Method development: artificial flower design

Experiment 1: do bees use sight when pollen foraging in open flowers?

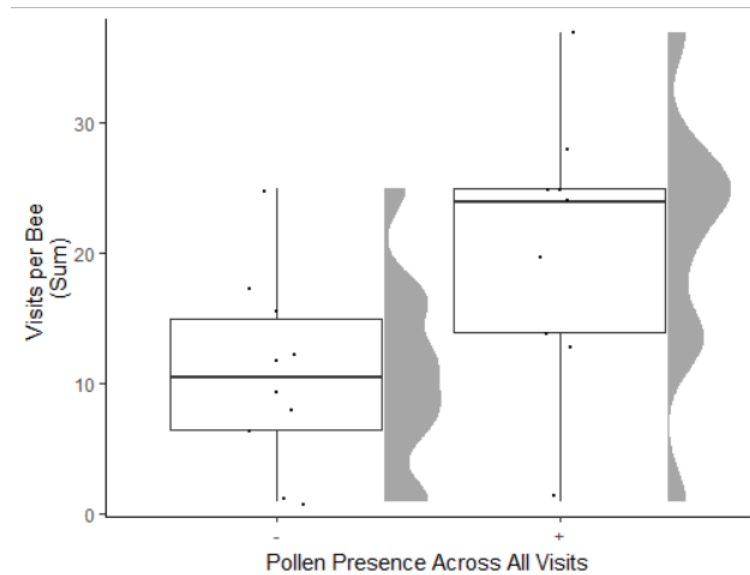
Aim – Determine whether bees will use visual cues when foraging for pollen.

To assess whether bees can detect the pollen surrogate used, *Lycopodium* spores, we assessed bee visitation rates to flowers with and without reward. Flowers consisted of a 38mm diameter yellow card disc with a white pipe cleaner emerging 10mm from the centre of the disc (Little Crafty Bugs Ltd., UK: product codes CV/CARD-21112 and CV/PC-51619, respectively) holding approximately 25mg spores. 5µl linalool solution (1:100,000 linalool:hexane by volume) was added to the cardboard disc to facilitate visitation as in Chapter Two. Four flowers of each treatment were presented randomly in vertical a 3×3 grid, leaving the middle position empty (Appendix 12A), with 100mm vertical and horizontal separation on the wall opposite the colony entrance. Bee visitation preference was assessed.



Appendix 12A. Equal numbers of rewarding and unrewarding flowers were randomly positioned in an array of artificial flowers.

Eighteen bees from two colonies undertook 294 visits. Bees were more likely to visit rewarding than unrewarding flowers (Appendix 12B; $6.3 \pm 8.7\sigma$ and $10.4 \pm 15.1\sigma$ visits per bee respectively). This demonstrated the need to develop a flower design which enclosed the reward.



Appendix 12B. Summary statistics show bees do not equally visit flowers with (+) and without (-) pollen surrogate (*Lycopodium* spores) with a greater number of visits to flowers with rewards.

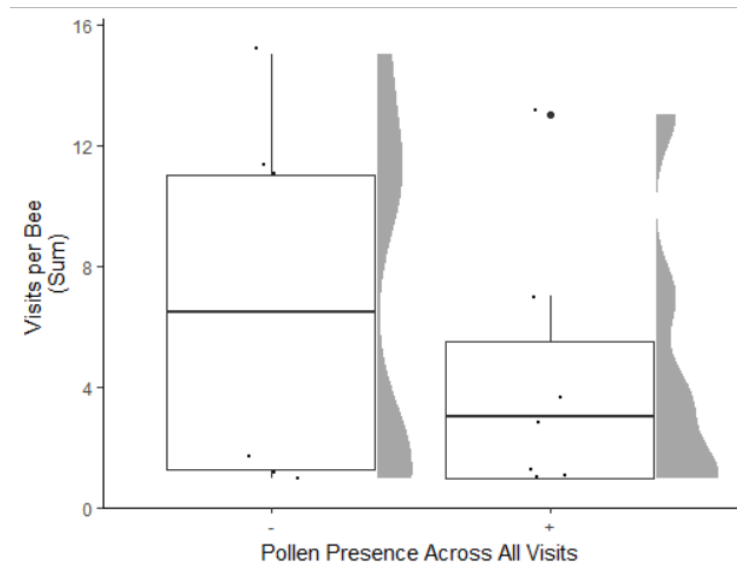
Experiment 2: Enclosed pollen-dispensing artificial flowers

Aim – Develop pollen-dispensing artificial flowers (starting from Russell & Papaj, 2016).

As bees appeared able to detect the presence of pollen surrogate on artificial flowers, an enclosed floral design was developed and bees' preferences for enclosed flowers with and without pollen was assessed (Figure 19). Artificial flowers consisted of a plastic tube (50mm deep, 25mm) painted grey, with a yellow plastic corolla (38mm diameter (Little Crafty Bugs Ltd., UK: product EF/FMA4-1055407, respectively)) and artificial anther containing pollen surrogate consisting of 20mm long pipe cleaner. Flowers were attached to a grey painted dowel to allow horizontal presentation. Bees were presented a choice of two conditions: (1) empty flowers, and (2) pollen flowers of equal numbers. Eight flowers were positioned randomly in an 8×8 array. As bee visitation was again very low 5µl linalool solution (1:100,000 linalool:hexane by volume) was added within artificial flowers at the base of the "tube". Bees were allowed to forage in the array individually. After a flower was visited it was replaced with a new one of the same treatment in a random location.

Seven bees from two colonies undertook 44 visits. There was little difference between rewarding than unrewarding flowers (Appendix 12C; $6.8 \pm 6.2\sigma$ and $4.3 \pm 4.2\sigma$ visits per bee respectively) and low visitation rates made it necessary to further improve bee visitation of artificial flowers.

Appendices



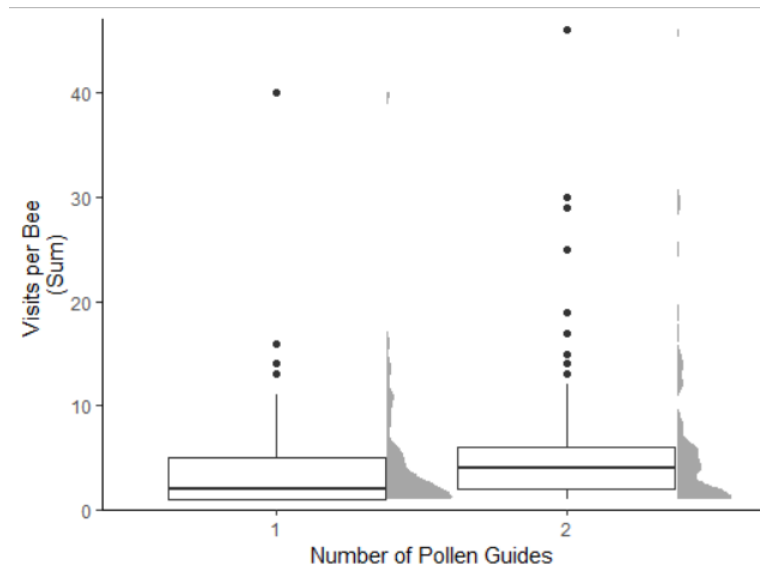
Appendix 12C. There was little difference in visitation rates to enclosed design flowers with (+) and without (-) pollen surrogate.

Experiment 3: Pollen Guides

Question – Can flower visitation be improved by adding pollen guides?

Bee visitation to artificial flowers was low and few bees entered the flower tube thus remaining unrewarded, so pollen guides were added. Pollen guides consisted of a straight line of yellow paint (Flash Gitz Yellow, Citadel Colour), approximately 5mm wide, painted from the mouth to the base of the artificial flower tube. Bees were presented with 1 or 2 guides (180° from one another) which were balanced across scent treatments. The flowers and methods from preliminary experiment 2 were used again here.

Fifteen bees from two colonies undertook 143 visits. Two pollen guides were more effective than one (Appendix 12D; $6.4 \pm 8.2\sigma$ and $4.4 \pm 6.0\sigma$ visits per bee respectively), increasing bee visitation by 46.8% (GLMM: Z-value=6.434, $P < 0.001$) and were therefore adopted.



Appendix 12D. Bees preferred artificial flowers with two pollen guides over one ($P < 0.001$).

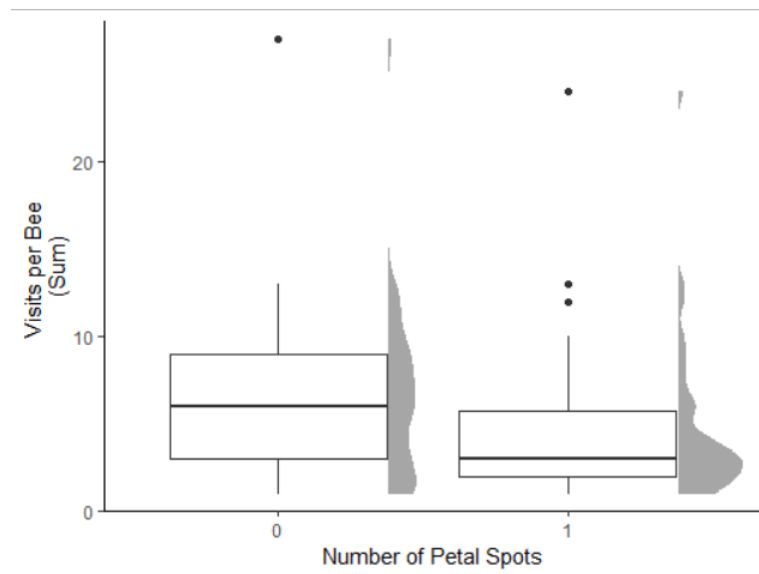
Experiment 4: Petal Spots

Question: Can visitation be improved using petal spots?

It was observed that bees appeared attracted to flowers containing other bees, as well as dark areas of the array (i.e., unused Velcro tabs). Dark spots on flower corollas have been suggested to be pollinator mimics (Schiestl, 2010b) which improve flower visitation through social learning (Goulson *et al.*, 2013) which is well documented in *Bombus* species. Spots (approximately 8mm diameter) were added to corollas of 50% of the artificial flowers in arrays, balanced across treatments, with black paint (Abaddon black, Citadel Colour). The flowers and methods from preliminary experiment 2 were used again here.

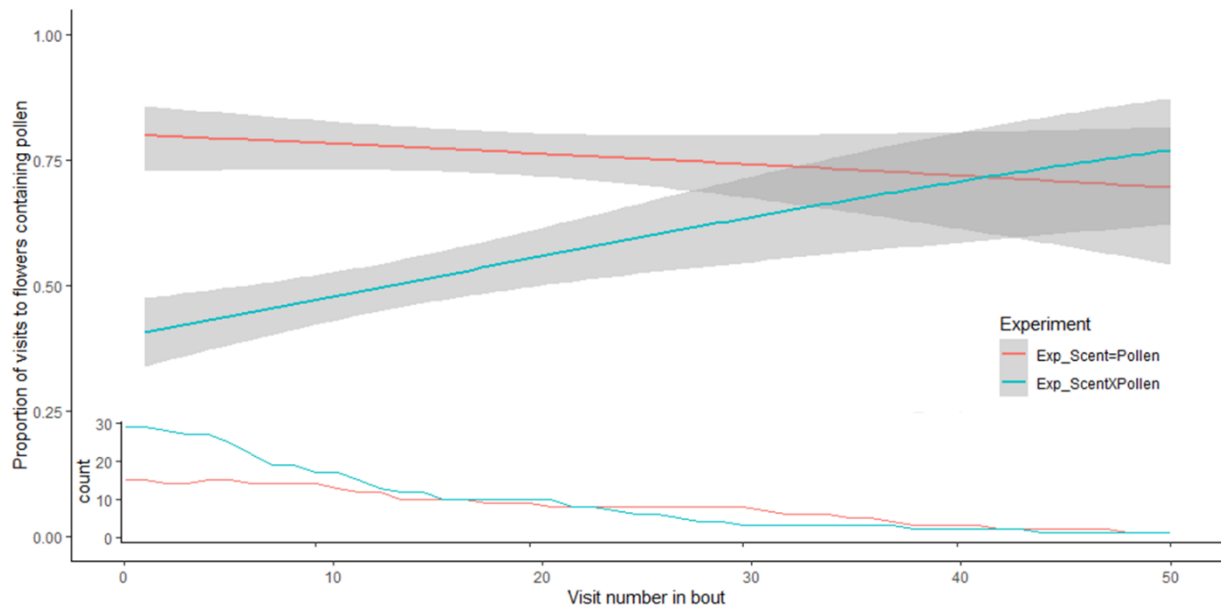
Twelve bees from two colonies undertook 134 visits. Bees visited flowers with corolla spots less than those without spots (Appendix 12E; $6.7 \pm 5.0\sigma$ and $4.68 \pm 4.4\sigma$ visits per bee respectively), reducing visitation by 27.4% (GLMM: Z-value=6.434, $P < 0.001$). Spots were abandoned.

Appendices



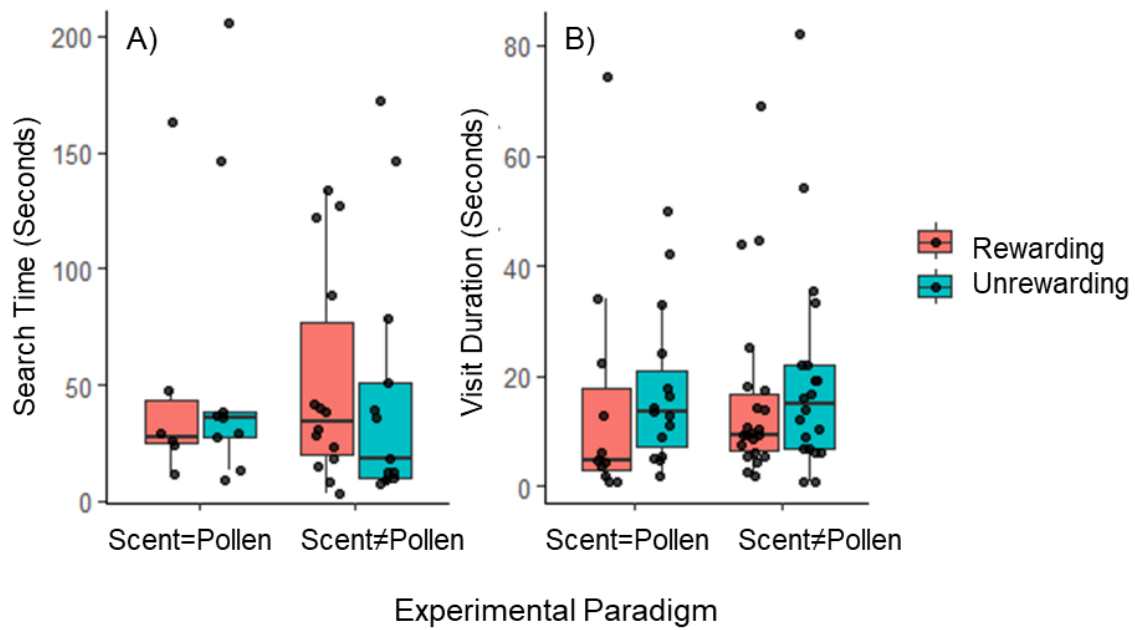
Appendix 12E. Addition of a petal spot decreased flower visitation by 27.4% ($P < 0.001$).

Appendix 13. Flower preference line graph using raw data



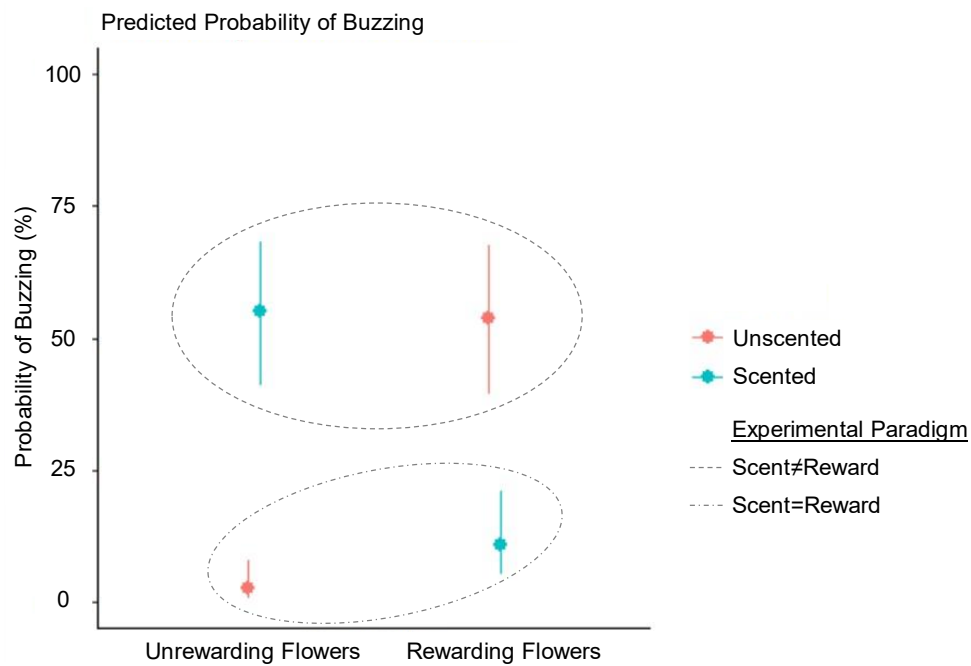
Appendix 13. The proportion of bees selecting rewarding flowers in the two experimental treatments. Bees initially used scent to select rewarding flowers in both treatments but improved their foraging success with experience. This figure contrasts with Figure 20 in the main text which plots the probability of bees selecting rewarding flowers calculated from the binomial regression.

Appendix 14. Bee search time and visit duration



Appendix 14. Bees spent equal time searching for flowers (A) and foraging within flowers (B), regardless of the presence of reward, scent or experimental treatment ($P > 0.05$).

Appendix 15: Buzzing visits model predictions



Appendix 15. Unrewarding unscented flowers were buzzed significantly less 7.7% (± 2.1 of visits with all buzzes produced by two bees) than all other treatments. Flowers which contained only scent or pollen received equal buzzes (49.1% ± 32.3 and 51.3% ± 32.6 respectively). Flowers which were both rewarding and scented received buzzes on 12.4% of visits (± 17.8). This presents probabilities calculated from the binomial GLMM contrasting with Figure 21 which presents the raw data.

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Appendix 16: buzzing visits binomial GLMM output

The likelihood of bees buzzing when visiting flowers in each treatment was analysed with binomial GLMMs (buzzing visits coded = 1, non-buzzing visits = 0) as the scent-reward pairs were not equivalent we analysed them separately. Scent and visit were fitted as fixed effects. A nested random effect structure with bee identity within colony estimated zero effect at the colony level which was therefore dropped ($\text{Buzzing} \sim \text{Scent} + \text{Visit} + (1 \mid \text{Bee ID})$) and assumptions were checked in DHARMA. The likelihood of bees buzzing when visiting artificial flowers was analysed with binomial (logit linked) GLMMs. Significant values are in bold.

Treatment: Scent=Reward

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) ['glmerMod'] Family: binomial (logit) Formula: <i>Buzzing</i> ~ <i>Scent</i> + <i>Visit</i> + (1 <i>Bee ID</i>)					Analysis of Deviance Table (Type III Wald chisquare tests)		
Fixed effects:	Estimate	Std. Error	z-value	Pr(> z)	Chisq	Df	Pr(>Chisq)
Intercept	-4.96909	1.02161	-4.864	<0.001	23.658	1	<0.001
Scent	1.80514	0.51698	3.492	<0.001	12.192	1	<0.001
Visit	0.03286	0.01484	2.214	0.027	4.901	1	0.027

Marginal R^2 (proportion of variance explained by only the fixed effects): 0.079.

Conditional R^2 (proportion of variance explained by the whole model (fixed and random)): 0.635.

Treatment: ScentXPollen

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) ['glmerMod'] Family: binomial (logit) Formula: <i>Buzzing</i> ~ <i>Scent</i> + <i>Visit</i> + (1 <i>Bee ID</i>)					Analysis of Deviance Table (Type III Wald chisquare tests)		
Fixed effects:	Estimate	Std. Error	z-value	Pr(> z)	Chisq	Df	Pr(>Chisq)
Intercept	0.228741	0.289088	0.791	0.429	0.6261	1	0.4288
Scent	0.016503	0.243051	0.068	0.946	0.0046	1	0.9459
Visit	-0.005169	0.011432	-0.452	0.651	0.2045	1	0.6511

Marginal R^2 (proportion of variance explained by only the fixed effects): 0.001.

Conditional R^2 (proportion of variance explained by the whole model (fixed and random)): 0.236.

Appendix 17. Follow-up experiments

Experiment 1: Honest signalling

Rationale

Foraging bees benefit from being able to discriminate between rewarding and unrewarding flowers. Honest signals are those which correlate with a flowers reward (e.g. quantity or quality). Pollen-only flowers can change their scent in response to visitation but it is unknown whether pollen foraging insects will use these signal changes to inform foraging decisions.

Experimental design

Bees would be trained to associate pollen reward with one of two scents and then their preference changes tested when the trained rewarding scent becomes no longer rewarding. Training would occur by placing pollen in the flight arena in a 60mm diameter petri dish placed on filter paper treated with either linalool or limonene and individual foragers allowed 2 foraging bouts to collect pollen from the array.

Treatment	Training VOC	Experimental VOC with Pollen	Experimental VOC without Pollen
Honest	Linalool	Linalool	Limonene
Dishonest	Linalool	Limonene	Linalool
Honest	Limonene	Limonene	Limonene
Dishonest	Limonene	Linalool	Linalool

Experiment 2: Uncertainty of reward in a patch

Rationale

Bees foraging in natural environments must choose between groups of flowers in a population, some of which are rewarding and others which are unrewarding (i.e. due to previous foraging visits, or due to individual plants which would increase pollen import/export by signalling when they remain unrewarding).

Experimental design

Bees would be trained to associate pollen reward with one of two scents which are always rewarding, then assess bees responses to changed reliability of reward. Bees would be trained to associate one of two scents with pollen by allowing them to forage on artificial flowers all of which are rewarding for 2 bouts. Bees would then be tested by changing the reliability of flowers (% of

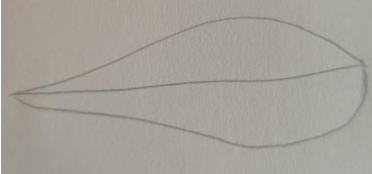
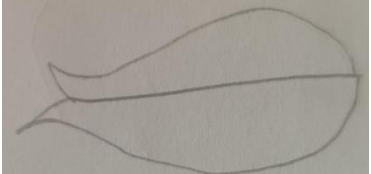
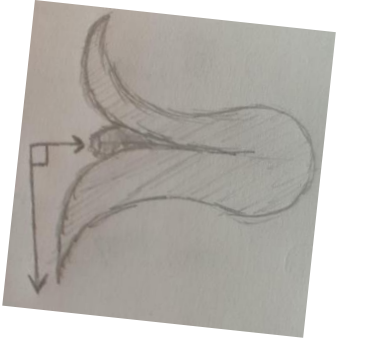
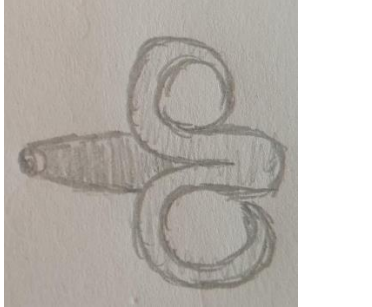
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flowers containing reward) and their preferences and flower switching would be assessed. A range of reliabilities would be tested.

Trial	Scent	Training Reliability	Test Reliability
1	Linalool	100	80
	Limonene	0	20
2	Linalool	100	50
	Limonene	0	50
3	Linalool	100	20
	Limonene	0	80

Appendix 18: *Echeandia luteola* pollination ecology

The monocotyledon *Echeandia luteola* was found at one field site, San Isidro Ochil. Flowers of this species remained closed throughout morning floral observations, when greatest floral visitation was observed in other focal species, opening later in the day. Therefore, detailed observations were made of this species to plot floral opening over one day. Five individuals of this species were found, four of which had just one flower spike, the fifth having a branched flower spike presenting flowers on five branches (A-D). Observations were made hourly from 09:30 until flowers began to open, from which time observations were made every 30 minutes until dusk at 18:30. Petal positions were characterised as either: closed, starting to open, anthers visible, petals 90°, partly reflexed and fully reflexed (F). Floral visitor observations were made for 10 minutes at every observation time.

A)	B)	C)
		
D)	E)	F)
		
Appendix 18. <i>Echeandia luteola</i> petal positions were characterised as: (A) closed, (B) starting to open, (C) anthers visible, (D) petals 90°, (E) partly reflexed and (F) fully reflexed		



Result

In total, 15 flowers were observed opening on four (of five observed) observed plants

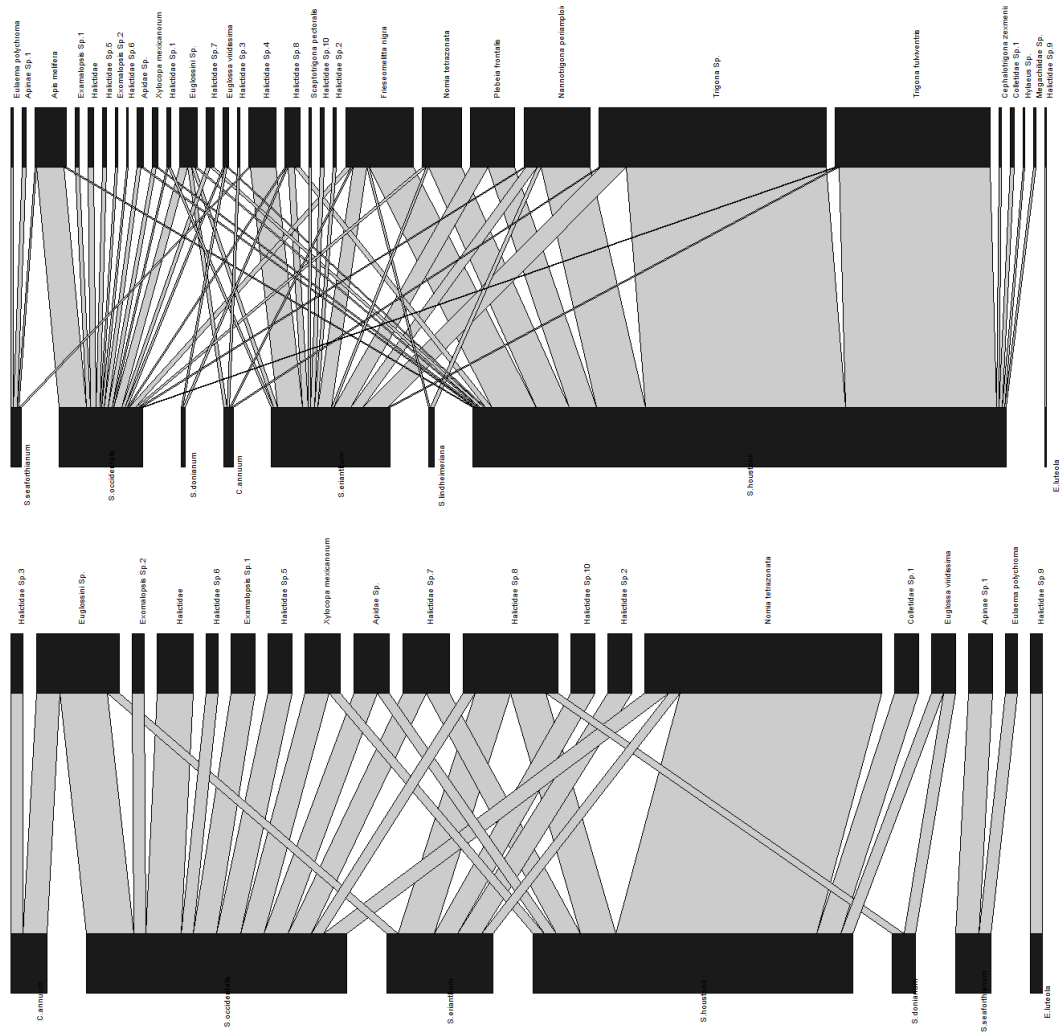
Only bees were observed visiting *Echeandia luteola* was observed.

“At 15:35 Small bee (#7 of day 22/X/2019) landed and buzzed 1 flower. Very quick land and take-off. Then harassing #8 as they visit flower. Male and Female of same species? Male takes a viewpoint on leaves over the lilly and harasses any other bee which lands.”

Number of *Echeandia luteola* flowers observed opening.

Plant	Flower Spike	No. Flowers
1	-	2
2	A	3
2	B	2
2	C	3
2	D	0
3	-	2
4	-	3
5	-	0

Appendix 19: All interactions network analysis



Appendix 19, Fig1. The plant-pollinator interaction network and buzz pollination sub-network of all observed interactions. Exported in 1500*700

Network Metric	Visitation Network				Buzz Pollination Sub-Network				
	Null Model		Observation		Null model		Observation		
	95% CI Lower	95% CI Upper	Value	Z- Score	95% CI Lower	95% CI Upper	Value	Z- Score	Dir' of Δ
Pollinator Niche Overlap	0.58	0.58	0.33	-5.79	0.36	0.56	0.28	-3.60	-
Plant Niche Overlap	0.32	0.65	0.12	-4.32	0.20	0.54	0.10	-2.90	-
H ₂ '	0.05	0.11	0.47	23.49	0.15	0.34	0.56	6.54	+
Weighted NODF	41.2	53.5	22.2	-8.03	15.0	28.7	13.83	-2.29	-
Connectance	0.25	0.29	0.24	-3.20	0.26	0.32	0.23	-4.65	=
Links Per Species	1.62	1.87	1.54	-3.20	1.35	1.62	1.15	-4.65	-
Alatalo interaction evenness	0.45	0.52	0.44	-1.98	0.62	0.79	0.54	-3.70	+

