Effects of floral morphology on plant-pollinator interactions in buzz-pollinated flowers

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Declaration

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.

Signature of candidate:

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The Lilacs bending many a year
Will sway with purple load
The Bees will not despise the tune
Their Forefathers have hummed

Emily Dickinson

Abstract

The remarkable diversity of floral forms across angiosperms is largely shaped by interactions between plants and pollinators. Buzz-pollinated flowers have a tight relationship with bees, their primary pollinators, as they require externally produced vibrations for pollen release. These flowers have evolved various morphologies during this relationship, many of them variations on the same theme, yet the functional significance of these forms remains understudied. In this thesis, I examine the effects of different buzz-pollinated morphologies on plant-pollinator interactions. The species I studied vary in stamen arrangement and stamen dimorphism. First, I compared vibration transmission between flowers with contrasting stamen arrangements using artificial vibrations applied to a focal anther. Second, I used cantilever theory and displacement experiments to calculate the natural frequency of two different stamen types in six *Solanum* taxa, which differ in size and mating system. Third, I assessed bumblebee buzz characteristics on two of these species, to find out whether they match their natural frequency to each flower type. Finally, I asked whether the role of floral orientation differs between radial and bilateral buzz-pollinated flowers, by manipulating their orientations and measuring bumblebee-mediated pollen deposition on the stigmas. I found that the transmission of vibrations varies with stamen arrangement: more uniformly arranged flowers transmit vibrations more faithfully than non-uniform flowers and some arrangements promote the amplification of vibrations. I also found that stamen natural frequency differs between plants with differing mating systems. Bees do not appear to adjust their buzzing frequencies to match these natural frequencies, although they may respond to differences in pollen release. Last, I found that the horizontal orientation of bilateral flowers may function to increase pollen transfer in outcrossing flowers. These findings shed light on key biomechanical and behavioural aspects of buzz pollination and point to potential functions of different floral morphologies.

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Statement of contribution by collaborators

For Chapter Two, plants were grown in the US by Dr Avery Russell (section 2.3.1), who also contributed the photos for Figure 2.1. Plant experiments and filming were performed by Dr Karl Foord (sections 2.3.2 and 2.3.3). All data extraction and analysis from this video footage was carried out by Lucy Nevard.

Chapter One: General introduction

The first flowers bloomed approximately 140-250 million years ago, beginning the angiosperm takeover of plant life (Ramshaw et al., 1972; Donoghue, 2019). The exact morphology of these early flowers is debated, but they were likely radially symmetric, with unfused petals and relatively exposed reproductive organs (Friis et al., 2006; Doyle, 2012; Sauquet et al., 2017). Since their first appearance, flowers have assumed a remarkable diversity of forms, involving evolutionary innovations such as fused organs, bilateral floral symmetry, and specialised reproductive morphologies (Hodges, 1997; Endress, 1999; Phillips et al., 2020). Floral traits mediate plant-pollinator interactions in zoophilous plants (Fenster et al., 2004; Johnson et al., 2006), and are shaped by them, driving floral diversity (Vamosi and Vamosi, 2010; van der Niet and Johnson, 2012). An extreme example of this is the diffuse co-evolution of the long nectar spur of the star orchids (*Angraecum* spp.) with the long tongue of the hawkmoths *Xanthopan* spp. in Madagascar (Nilsson, 1988; Wasserthal, 1997; Harder and Johnson, 2009; Netz and Renner, 2017). The existence of an extremely long-tongued pollinator was posited by Darwin when he saw specimens of the long-spurred orchid (Darwin, 1862) and only discovered decades later (Rothschild and Jordan, 1903). . Trait-matching between flower and pollinator remains central to pollination research, yet there are even subtler ways in which floral morphology can affect plant-pollinator interactions.

1.1 The poricidal anther

The vast majority of angiosperms rely on animal vectors, primarily insects, for pollination (Klein et al., 2007; Ollerton et al., 2011). Pollen consumption by these insects, particularly bees, creates a conflict between plant and pollinator (Harder and Thomson, 1989; Klinkhamer and de Jong, 1993; Thomson, 2003). Bees are motivated to collect as much pollen as possible, whilst plants are driven to minimise the proportion of pollen consumed, and maximise the chances of pollen transfer (Harder and Thomson, 1989; Van Der Kooi et al., 2021). Furthermore, because plant male fitness increases with the number of visitors transferring pollen, it decreases with the amount of pollen removed by an individual bee (Harder and Thomson, 1989; Harder and Wilson, 1994; Van Der Kooi et al., 2021). Flowers have evolved numerous strategies in this conflict, including the attraction of visitors, such as non-pollinivorous taxa, with nectar (Pyke, 2016; Parachnowitsch et al., 2018), or the production of defence compounds in pollen to discourage pollen consumption (Junior et al., 2011; Wang et al., 2019; Rivest and Forrest, 2020). Morphological innovations have also arisen, such as the keel flower, a highly modified corolla found in Fabaceae and other families, which functions to brush concealed pollen onto the ventral side of the nectar-foraging visitor.(Harder and Wilson, 1994; Westerkamp, 1997). Such morphologies can lead to tight relationships between flower and bee, as seen between some species of the highly specialised *Blumenbachia* and their only effective pollinators, colletid bees (Siriani-Oliveira et al., 2020).

The conflict over pollen is most intense in flowers without nectar or other rewards such as oils (Simpson and Neff, 1981). These flowers must reward visitors with pollen whilst minimising the loss of costly male gametes to consumption (Westerkamp, 1996), resulting in floral strategies to lessen the efficiency of bee foraging. One of the most successful of these strategies is to conceal the pollen inside the anther, which is modified from longitudinally dehiscent (exposed pollen) to a poricidal, tube-like structure in which pollen is released only from small, apical pores (Matthews and Maclachlan, 1929; Michener, 1962; Buchmann, 1983). Crucially, these flowers are obligately buzz pollinated; bees must perform vibrations on the flower to efficiently collect pollen (Rick, 1950; Michener, 1962; Macior, 1964; Buchmann, 1983; Corbet et al., 1988; Gottsberger and Silberbauer-Gottsberger, 1988; Endress, 1997). A buzzing bee typically curls her body around the stamens, grasps the base with her mandibles and uses her indirect flight muscles to produce vibrations, which are transferred via her head to the anthers (Buchmann, 1983; King et al., 1996; Vallejo-Marín, 2019). Pollen is expelled from the anther pores onto the bee’s body, from where it can be groomed and packed, or inadvertently transferred to another flower (Buchmann, 1983; Vallejo-Marín, 2019). The pollen grains of poricidal flowers are also adapted to this form of release, being small, numerous, and dry (generally lacking pollenkitt) (Buchmann, 1983; Faegri, 1986; Corbet et al., 1988), enabling their ejection from the anthers in diffuse clouds (Buchmann and Hurley, 1978; Amorim et al., 2019). Notable exceptions include the relatively sticky pollen of kiwifruit (*Actinidia deliciosa*) (King and Lengoc, 1993) and the thread-linked pollen of rhododendrons (*Rhododendron* spp.) (King and Buchmann, 1995; Song et al., 2019). The poricidal anther first emerged at least 55 million years ago, the date of the first known fossil (Friis et al., 2006), but has evolved independently within many plant lineages (Vogel, 1978; Buchmann, 1983; Faegri, 1986; Mast et al., 2004; De Luca and

Vallejo-Marín, 2013), and provides an excellent example of convergence in morphology and function. Around 6% of angiosperm species have poricidal anthers, within at least 72 families, across both dicots and monocots (Buchmann, 1983; Proença, 1992; Vallejo-Marín et al., 2010), and including many economically important crops, such as tomatoes, aubergines, and blueberries (Bobiwash et al., 2018; Cooley and Vallejo-Marín, 2021). Poricidal dehiscence can be achieved via different mechanisms across lineages (Hermann and Palser, 2000; Marazzi et al., 2007; Carrizo Garcia et al., 2008; Cortez et al., 2014), and some intriguing buzz-pollinated species possess anthers intermediate between longitudinally dehiscent and poricidal, as seen in Myrtaceae and Fabaceae, representing perhaps a transitional stage in their evolution (Proença, 1992; Marazzi et al., 2007; Carrizo Garcia et al., 2008). In a few taxa, the corolla itself effectively functions as a poricidal anther, creating a tube around the stamens, as in buzz-pollinated *Pedicularis* spp. (Corbet and Huang, 2014; Amorim et al., 2019). However, the effect of different types of buzz-pollinated morphologies, including intermediate anther dehiscence or corolla modifications, has yet to be explored.

The repeated evolution of the poricidal morphology and its tight association with buzz pollination points to its advantages as a reproductive strategy. One benefit of the poricidal anther is its function in pollen dispensing: bees typically buzz flowers multiple times in one visit (Buchmann and Cane, 1989; De Luca et al., 2013; Russell et al., 2016b), but the amount of pollen they can remove is restricted (Harder and Barclay, 1994; King and Buchmann, 1996), and pollen remains in the anther even after dozens of vibrations, although this release pattern varies among species (Brito et al., 2021; Kemp and Vallejo-Marín, 2021). This potentially allows flowers to distribute pollen to multiple visitors, increasing male fitness potential (Harder and Thomson, 1989; Harder and Barclay, 1994; Harder and Wilson, 1994). The dispensing hypothesis is also supported by evidence that older virgin flowers release pollen at higher rates than younger flowers in both *Dodecatheon conjugens* (Harder and Barclay, 1994) and *Senna reticulata* (Dellinger et al., 2019a), increasing the chances of reproduction, although this age effect is not universal (Dellinger et al., 2019a). Poricidal flowers can also increase visitation rates by the deception of visitors: bright yellow, UV-absorbing anthers act as pollen mimics, attracting bees, while actual pollen availability remains cryptic (Vogel, 1978; Lunau, 2005). Similarly, chemical cues from concealed pollen are less reliable than when pollen is exposed, further decreasing the ability of the bee to detect pollen availability (Dobson et al., 1999; Russell et al., 2018). The visitation of bees to flowers with potentially empty anthers aids female fitness by increasing the chances of stigma contact.

Buzz-pollinated plants can also benefit from restricting pollen access to a small subset of potential visitors, reducing reproductive interference such as interspecific pollen transfer, as in other specialised pollination systems (Stebbins, 1970; Fenster et al., 2004; Morales and Traveset, 2008). There are three levels of restrictions in operation here. First, bees (Anthophila) are the only insect group which regularly performs floral vibrations (Buchmann et al., 1977; Vallejo-Marín and Vallejo, 2021). Second, only a subset of bees are able to buzz flowers, notably excluding the genus *Apis* (King and Buchmann, 2003; Cardinal et al., 2018; Vallejo-Marín, 2021). The inability of *Apis* to perform floral vibrations has consequences for buzz-pollinated plants in geographic regions where *Apis mellifera* is introduced and in competition with native pollinators, such as the Americas (Moritz et al., 2005). Last, the nectarlessness of most buzz-pollinated flowers restricts visits mostly to pollen foragers. This may be especially relevant for those plants pollinated by social bee species with task allocation between individual workers, e.g. *Bombus* (Free, 1955; Hagbery and Nieh, 2012). Although nectarlessness could further reduce the chances of reproductive interference, some poricidal lineages have secondarily gained nectaries (Dukas and Dafni, 1990; Vallejo-Marín et al., 2010; Moquet et al., 2017), potentially increasing pollinator attraction and altering their intrafloral behaviour (Tong et al., 2018). One of the few studies of the function of nectar in buzz-pollinated flowers showed that nectar foraging visitors positively impact plant reproductive success in Ericaceae (Moquet et al., 2017). In some pollinator environments, pollen limitation may thus outweigh reproductive interference as a driver of floral traits.

The poricidal anther is not a fool proof strategy for pollen concealment: pollen theft, removal of pollen without stigma contact, from poricidal flowers is common (Solís-Montero et al., 2015; Staines et al., 2017; Mesquita-Neto et al., 2018). Potential pollen thieves include buzzing bees who are too small to touch the stigma whilst buzzing (Buchmann, 1983; Solís-Montero and Vallejo-Marín, 2017; Mesquita-Neto et al., 2018), or non-buzzing bees who chew the anthers (Renner, 1983), glean pollen from the anther pores (Anderson and Symon, 1988) or collect pollen left over from visits by previous bees (Snow and Roubik, 1987). Although many of these bees are small, like the widespread thief *Trigona* spp (Mesquita-

Neto et al., 2018), and individual foragers may not collect much pollen, the proportion of mating opportunities and male gametes lost in this way can be high in environments with a high ratio of thieves to legitimate pollinators (Rego et al., 2018).

1.2 Buzz-pollinated morphologies

Buzz-pollinated plants share key floral traits central to their relationship with buzzing bees. The corolla is usually flat or reflexed and the androecium fully exposed (Buchmann, 1983; Faegri, 1986), although there are exceptions, like the campanulate genus *Vaccinium* (Hermann and Palser, 2000). Stamens consist of relatively short filaments and long poricidal anthers and are brightly coloured (Faegri, 1986; Dellinger et al., 2019b), in concordance with their role in pollinator signalling (Russell et al., 2018). Here, I introduce two, interrelated ways in which floral morphology can differ between buzz-pollinated flowers: the arrangement of stamens in the flower, and the relative size and function of these stamens, collectively known as stamen architecture (Endress, 1996; Jeiter et al., 2020). Given that stamens are the site of pollen release and the primary structures with which visitors interact, differences in stamen arrangement and morphology between and within flowers are expected to impact plant-pollinator interactions.

Researchers have repeatedly observed that buzz-pollinated flowers across diverse groups share a strikingly similar morphology, in which flowers are pendant and radially symmetric, and stamens are centrally arranged in a cone shape around the style (Macior, 1964; Faegri, 1986; Harder and Barclay, 1994) (Figure 1.1A and B). Faegri (1986) coined this the "solanoid flower" after its prevalence in the genus *Solanum* (Endress, 1997; Glover et al., 2004), and this flower type remains archetypal in buzz pollination studies. The anther cone has convergently evolved many times, including in the families Boraginaceae, Liliaceae, Melastomataceae, Primulaceae, Rubiaceae Solanaceae (Almeda, 1977; Faegri, 1986; Puff et al., 1995; Glover et al., 2004; Vallejo-Marín et al., 2010). Glover (2004) shows that even in closely related species, cones can be formed via distinct mechanisms: anthers are held together by trichomes in the tomato *Solanum lycopersicum* (Figure 1.1B) and by extracellular secretions in *S. dulcamara* (Figure 1.1A). Other taxa are instead fused via the filaments (Schwartz-Tzachor et al., 2006) or have anther cones without physical attachment between the stamens such as *Borago officinalis* (Corbet et al., 1988) or *Solanum seaforthianum* (pers. obs.). The prevalence of this flower form in buzz-pollinated lineages suggests that it represents an adaptive peak (Harder and Barclay, 1994; Vallejo-Marín et al., 2021), yet its function in plant-pollinator interactions has been little studied. One function could be to constrain bee position: bees are obliged to wrap their body around the entire cone, ensuring contact with both anther and stigma (Glover et al., 2004). The close proximity of anther pores to each other may also help to direct pollen in a particular direction, e.g., a specific area of the bee body (Harder and Barclay, 1994; Glover et al., 2004).

The drivers of a looser stamen arrangement in some lineages are also unclear, although species with loose anthers are common, particularly in the largely buzz-pollinated family Melastomataceae (Dellinger et al., 2019b; Gavrutenko et al., 2020) and within *Solanum* (Figure 1.1C and D). Critically, a looser arrangement increases herkogamy, the degree to which the stigma and anthers are spatially separated, further reducing the chances of self-pollination and increasing the plant's dependence on pollinators (Barrett, 2002; Hargreaves et al., 2009; Opedal, 2018). Because flower size varies among buzz-pollinated species, herkogamy also paves the way for increased specialisation on subsets of pollinators through size-matching (Solís-Montero and Vallejo-Marín, 2017; Mesquita-Neto et al., 2021).

A looser stamen arrangement also allows the evolution of heterantherous flowers, in which stamens are morphologically distinct and often spatially separated, with corresponding differences in function (Müller, 1883; Vallejo-Marin et al., 2009) ((Figure 1.1E and F). Heteranthery is strongly associated with buzz pollination (Vogel, 1978; Jesson and Barrett, 2003; Vallejo-Marin et al., 2009) and is particularly well studied in Fabaceae (Luo et al., 2009; Pinheiro-Costa et al., 2018), Melastomataceae, in which it is widespread (Dellinger et al., 2019a; Melo et al., 2021), and *Solanum* (Vallejo-Marin et al., 2009; Papaj et al., 2017). Usually, there are two anther types: short, brightly coloured "feeding" anthers, which provide pollen for foraging bees, and fewer, longer, cryptic "pollinating" anther(s), which contribute mostly to pollination (Müller, 1883; Endress, 1997; Luo et al., 2009; Vallejo-Marín et al., 2010; Pinheiro-Costa et al., 2018; Dellinger et al., 2019a). They often differ in traits including pollen quantity (Luo et al., 2009; Pinheiro-Costa et al., 2018; Brito et al., 2021; Kemp and Vallejo-Marín, 2021), viability (Luo et al., 2008; Nepi et al., 2008), and release rates (Dellinger et al., 2019a; Telles et al., 2020; Brito et al., 2021). Some species have three anther types, such as in the legumes *Swartzia trimorphica* (Mansano and De

Souza, 2005), *Senna pendula* (Pinheiro-Costa et al., 2018), and *Cassia fistula* (Saab et al., 2021), although the functions of trimorphic stamens are not clear.

Heteranthery affects pollinator interactions in several fundamental ways. Heterantherous flowers are zygomorphic (bilaterally symmetric) relative to actinomorphic (radially symmetric) cone flowers (Bohs et al., 2007; Amorim et al., 2017). Bilateral symmetry can increase bee attraction (Rodríguez et al., 2004), affect their foraging behaviour (Culbert and Forrest, 2016), and creates a need for precise pollinator positioning (Gong and Huang, 2009). Heteranthery is also associated with an increase in herkogamy, often through enantiostyly or "mirror image" flowers, in which the style is deflected to the left or right, opposite to the pollinating anther (Jesson and Barrett, 2002; Vallejo-Marín et al., 2010). This promotes outcrossing, by reducing the chances of pollen transfer between flowers on the same plant, or geitonogamy (Jesson and Barrett, 2005). Additionally, the curved pollinating anther is often positioned such that it ejects pollen onto "safe sites" on the bee's body while she buzzes the feeding anthers (Koch et al., 2017; Mesquita-Neto et al., 2017). In some flowers, the zygomorphic corolla contributes to this division of labour by deflecting pollen from pollinating stamens onto the dorsal side of the bee (Amorim et al., 2017; Amorim et al., 2019). Heteranthery and division of labour between anthers reinforce the requirement for size-matched pollinators (Solís-Montero and Vallejo-Marín, 2017), and leaves flowers open to exploitation by bees buzzing the feeding anthers without contact with either pollinating anther or stigma, particularly smaller-bodied bees (Li et al., 2015; Papaj et al., 2017).

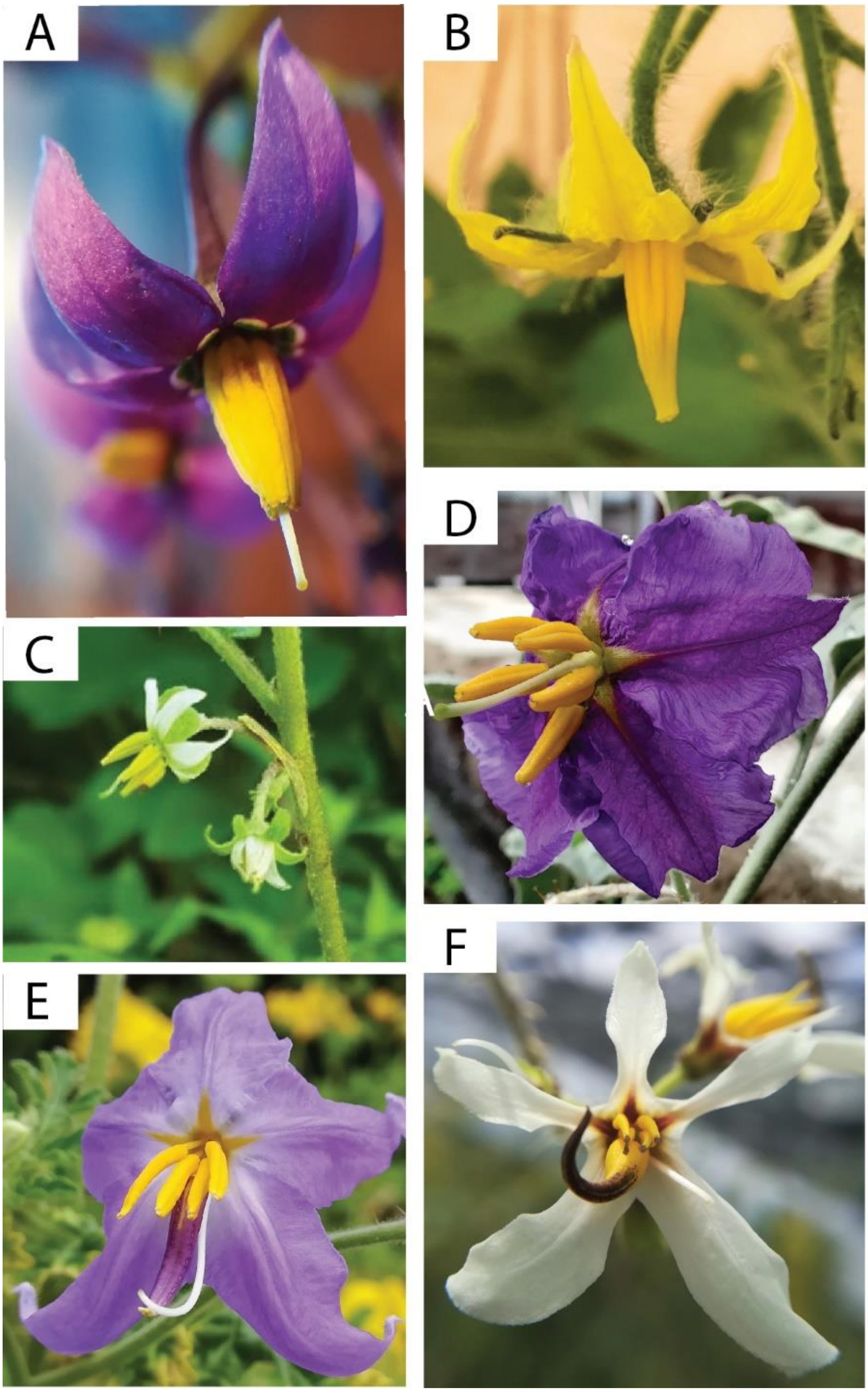

Figure 1.1 Examples of three floral morphology (stamen arrangement) types investigated in this thesis. Cone (A: *Solanum dulcamara*, B: *S. lycopersicum*); loose (C: *S. dasyanthum*, D: *S. eleagnifolium*); heterantherous (E: *S. citrullifolium*, F: *S. grayi grandiflorum*).

1.3 The biomechanics of buzz pollination

The tight relationship between vibrations and pollen release is the defining feature of buzz-pollinated flowers, yet the impact of floral morphology on vibrational properties has been little studied. Vibrations, including floral buzzes, can be characterised by three essential properties: duration, amplitude, and frequency (Cocroft and Rodríguez, 2005; De Luca and Vallejo-Marín, 2013; Mortimer, 2017). Single floral buzzes are typically between 0.1 and 2 seconds long and bees often produce multiple buzzes during a floral visit (Buchmann and Cane, 1989; De Luca and Vallejo-Marín, 2013) (Figure 1.2A). The amplitude of a buzz refers to its magnitude or intensity, and can be calculated as displacement, velocity or acceleration (Sueur, 2018; Rosi-Denadai et al., 2020). Vibrations also consist of multiple frequencies or harmonics, and the peak frequency, the "pitch", of a vibration has the highest magnitude: it contains the most energy (Sueur, 2018) (Figure 1.2B). In bee floral vibrations, the peak frequency is also the fundamental frequency (the first harmonic) and is typically between 100 and 400Hz (De Luca et al., 2019; Rosi-Denadai et al., 2020). This peak frequency contains most of the energy of the buzz, and higher harmonics likely contribute little to the interaction (Rosi-Denadai et al., 2020). Research on buzz pollination has largely focused on the frequency component of floral vibrations, partly because it can be accurately assessed with acoustic recordings, unlike amplitude (De Luca et al., 2018; Vallejo-Marín, 2019). However, the relationship between frequency and pollen release is tenuous: very high frequencies (outside the range of bee buzzes) may increase pollen release in some species (Harder and Barclay, 1994). Rosi-Denadai et al. (2020) characterised both frequency and amplitude effects and demonstrated that the relationship between frequency and pollen release is negative when amplitude is expressed as acceleration, but positive when it is expressed as velocity or displacement. Crucially, amplitude has a more straightforward positive relationship with pollen release: higher amplitudes cause more energy to be transferred to the anthers (Buchmann and Hurley, 1978; Harder and Barclay, 1994; King and Buchmann, 1996; De Luca et al., 2013; Rosi-Denadai et al., 2020; Kemp and Vallejo-Marín, 2021).

Substrate-borne vibrations are affected by substrate traits, as shown by the extensive research on plant-borne vibrations in the fields of insect communication and plant-herbivore interactions (Cocroft and Rodríguez, 2005; Cocroft et al., 2006). For instance, flexible plant stems attenuate vibrations more than stiff stems, leading to lower reliability of mating signals (Cocroft et al., 2006), and thicker leaves reduce the transmission and detectability of herbivore chewing cues (Velilla et al., 2020), which can stimulate plants to produce defence chemicals such as nicotine or anthocyanins (Pinto et al., 2019; Kollasch et al., 2020). Although there are few such investigations, floral vibrations are also affected by the substrate through which they travel, for example, they can be amplified as they travel through different floral organs from the base of a flower to the anthers (Brito et al., 2020). Species can also differ in their "coupling factor", a measure of amplitude attenuation (King, 1993; Arroyo-Correa et al., 2019), likely due to morphological and biomechanical differences between flowers. Additional floral structures, such as the connective appendages in the stamens of *Huberia bradeana* (Melastomataceae), may also aid pollination through their effect on the transmission of vibrations (Bochorny et al., 2021). In general, differences in biomechanical properties, and potentially pollen dispensing strategies, within and between flowers may be driving floral morphological traits, e.g. cone shape vs loose stamens, or heteranthery vs anther uniformity. For example, cone flowers may show a higher fidelity of vibration transmission compared to loose or heterantherous stamen arrangements. Similarly, stamen types in heterantherous flowers may differ in key biomechanical properties related to their division of labour. Yet few studies have compared the vibrational properties of different buzz-pollinated floral morphologies and their relationship with plant-pollinator interactions.

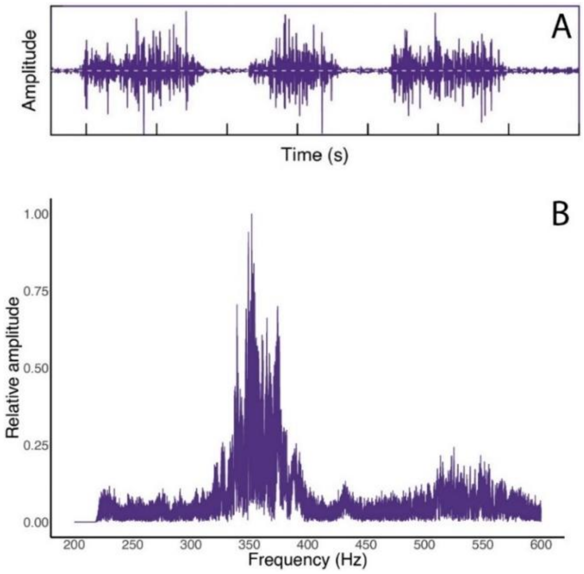

Figure 1.2 A: Oscillogram of a bumblebee floral vibration showing three buzzes. B: Frequency spectrum of the same vibration with a peak at 352 Hz.

1.4 Bee buzzing behaviour

Bees use their thoracic muscles to produce vibrations in a range of ecological contexts, including during flight (Burkart et al., 2011; Pritchard and Vallejo-Marín, 2020), communication with conspecifics (Larsen et al., 1986; Hrncir et al., 2006), and defence against predators (Hrncir et al., 2008; Pritchard and Vallejo-Marín, 2020; Vallejo-Marín, 2021). However, the use of thoracic vibrations to collect pollen (floral buzzes) is only present in 58% of all bee species across 15% of genera (Cardinal et al., 2018). Furthermore, although flies also collect pollen for their own consumption, there is only one published report of a fly, *Volucella mexicana*, buzzing a flower to release pollen (Buchmann et al., 1977; Vallejo-Marín and Vallejo, 2021). Floral buzzing behaviour has independently evolved 45 times within Anthophila, been secondarily lost 66 times (Cardinal et al., 2018) and predates the evolution of poricidal anthers (Proença, 1992; Cardinal et al., 2018), likely precipitating their evolution as a strategy to counteract high pollen loss. Bees will regularly buzz flowers with exposed pollen (Buchmann, 1985; Pellmyr, 1985; Russell et al., 2017), as it is more efficient than the main alternative, "scrabbling" (Russell et al., 2017). The disparity in buzzing ability between bee lineages could reflect trade-offs between different foraging behaviours, including on poricidal flowers – floral vibrations are efficient but energetically costly, whilst pollen theft through biting or gleaning might be more cost-effective for some bees (Russell et al., 2017; Mesquita-Neto et al., 2021).

Floral vibration characteristics can vary between and within bee taxa, driven somewhat by differences in size. Larger bees produce higher amplitude buzzes, across *Bombus* species (De Luca et al., 2014) and sometimes across individuals within species (De Luca et al., 2013), although this latter relationship is not consistently found (Nunes-Silva et al., 2013; Arroyo-Correa et al., 2019; Pritchard and Vallejo-Marín, 2020). Given that higher amplitudes release more pollen, larger bees may more efficient buzzers, which may contribute to observed differences in foraging behaviour between small and large taxa. The relationship between buzz frequency and body size is not linear, but larger-bodied species have a greater discrepancy between their flight frequency and floral vibration frequency, or "buzz ratio", being able to raise their floral buzz frequency high above their relatively low flight frequency (Burkart et al., 2011; De Luca et al., 2019). Within species, the effect of size on buzz frequency is inconsistent across studies (Nunes-Silva et al., 2013; Switzer and Combes, 2017; Rosi-Denadai et al., 2020).

The production of floral vibrations is an innate behaviour (Morgan et al., 2016; Russell et al., 2016b), but bee behaviour on buzz-pollinated flowers can shaped by floral traits, including chemical (Russell et al., 2017; Solís-Montero et al., 2018) and visual cues (Russell et al., 2018). In bumblebees, floral volatiles have been found to stimulate buzzing behaviour, whilst the presence of pollen on the anther (mechanosensory cues) has been found to suppress buzzing (Russell et al., 2017). Stamen architecture plays a key role in these interactions, including bee position and the choice of stamens to buzz. In cone flowers, bees must buzz all stamens simultaneously, whilst flowers with looser architectures allow bees to buzz individual stamens (Glover et al., 2004; Mesquita-Neto et al., 2018). Likewise, in heterantherous flowers, bees “stereotypically” buzz the attractive feeding anthers but are also highly flexible in their behaviour and regularly buzz the pollinating anther alone (Luo et al., 2008; Papaj et al., 2017) (pers.obs.).

The relationship between floral morphology and bee vibration characteristics is unclear. Several studies have compared bee vibrations on different plant species, with mixed results. *Bombus friseanus* buzz frequency varies between several *Pedicularis* species with different morphologies (Corbet and Huang, 2014), although this may be due to size assorting amongst individuals. *Bombus impatiens* individuals have been found to change their buzz frequency and duration between *Solanum* species of differing morphologies (Switzer and Combes, 2017). Individual bumblebees have also been shown to alter buzz amplitude between the closely related *S. citrullifolium* and *S. rostratum*, which differ in some morphological traits (Arroyo-Correa et al., 2019), perhaps due to differences in flower mass (Switzer et al., 2019). However, even in relatively similar flowers, it is hard to pin down the exact morphological or biomechanical traits that cause differences in buzzing of morphology on buzzing behaviour.

One possibility is that bees alter their buzzes in response to pollen release, which is itself related to floral morphology (Dellinger et al., 2019a; Kemp and Vallejo-Marín, 2021). Bees have been found to increase both frequency (Switzer et al., 2019) and amplitude (Russell et al., 2016b; Switzer et al., 2019) and reduce buzz duration (Russell et al., 2016b) on flowers which do not release pollen at all. The production of short, higher-energy buzzes may enable bees to assess quickly whether a flower is worth the time and energy spent continuing to buzz. Switzer et al (2019) found that bees did not tune their buzz frequency to the artificially-induced optimal pollen release frequency of their flowers, but rather always increased their buzz frequency in response to negative feedback (Switzer et al., 2019). These findings also raise the possibility that differing *rates* of pollen released can also trigger a response in the bee. Russell et al. (2016b). found that bees increase duration and amplitude within a single visit on a flower, indicating that diminishing pollen rewards may influence buzzing behaviour as bees increase their buzzing efforts to collect the last remaining pollen. Differences in pollen release patterns between flowers, and between stamens in heterantherous flowers, may thus influence bee vibrations (Brito et al., 2021; Kemp and Vallejo-Marín, 2021). Furthermore, bees learn associations between pollen rewards and floral traits (Muth et al., 2016; Russell et al., 2016a), however it is unclear whether this learning affects individual buzzing behaviour on different floral morphologies.

1.5 Thesis aims and structure

Floral morphology mediates interactions between plants and bees and has implications particular to the specialised buzz-pollinated system. In this thesis, I used a variety of approaches to investigate the effects of different buzz-pollinated morphologies on plant-pollinator interactions. The two main axes of variation I focus on are stamen arrangement (loosely held to tightly held) and degree of stamen dimorphism (weakly heterantherous to highly heterantherous). I assess their effects on several aspects of the interaction: the transmission of vibrations, biomechanical characteristics of stamens, bee buzzing behaviour, and the effect of floral orientation on pollen deposition.

My overall aims were to:

a) determine the effect of stamen arrangement on the transmission of vibrations in flowers, expecting that cone flowers will show higher fidelity of transmission than non-cone flowers.

b) calculate biomechanical properties of stamens in species with different levels of heteranthery, hypothesising that they vary with this difference in dimorphism, and between dimorphic stamens within flowers.

c) assess the behaviour of bees on flowers with different biomechanical properties, expecting that bees change their buzzing behaviour somewhat between substantially different plant species.

d) investigate how the relationship between heteranthery and floral orientation affects bee-mediated pollen deposition, hypothesising that a manipulation of orientation is detrimental to heterantherous flowers due to their requirement for precise pollinator positioning.

Below, I discuss these aims in more detail within the outline of each chapter.

Chapter two: Transmission of bee-like vibrations in buzz-pollinated plants with different stamen architectures

Buzz-pollinated plants require floral vibrations for effective pollen release from anther pores (Buchmann, 1983). Bees typically produce vibrations while grasping one or more anthers with their mandibles, and vibrations are transmitted to this anther(s), adjacent anthers, and the whole flower (Papaj et al., 2017; Vallejo-Marín, 2019). Buzz-pollinated taxa vary substantially in morphology, and these differences are likely to affect the transmission of vibrations, potentially impacting pollen release. However, the effect of floral morphology, e.g. stamen arrangement, on the transmission of vibrations has not been explicitly investigated. In this chapter, we compare vibration transmission between focal and non-focal anthers in four species with contrasting stamen architectures: cone (*Cyclamen persicum, Solanum dulcamara*), loose (*Exacum affine*), and heterantherous (*S. houstonii*). We used a mechanical transducer to apply artificial vibrations to focal anthers, measuring the vibration frequency and displacement amplitude at focal and non-focal anther tips simultaneously using high-speed video analysis (6,000 frames per second).

I ask the following questions:

1. Does the dominant frequency of vibrations change between focal and non-focal anthers in these flowers?
2. Does vibration amplitude (measured as displacement amplitude) change between focal and non-focal anthers?
3. Do vibration characteristics depend on plant species and/or the characteristics of the applied vibration?

Chapter three: Stamen natural frequency in six *Solanum* taxa with differing floral morphologies

Floral morphologies of buzz-pollinated flowers are related to biomechanical properties, which in turn affect their response to external forces, including floral vibrations (Niklas, 1992). A key property of all structures is their natural frequency. Vibrations of a stamen at its natural frequency are amplified through resonance (King and Buchmann, 1996; Timerman et al., 2014). Given the differences in stamen morphology within and between buzz-pollinated flowers, we also expect variation in natural frequency. As vibration amplitude is positively related to pollen release, this can impact on the pollen dispensing strategies of poricidal flowers and bees may theoretically exploit this resonance to maximise pollen collection (Rosi-Denadai et al., 2020; Kemp and Vallejo-Marín, 2021). The variation in stamen natural frequency between closely-related buzz-pollinated species has not so far been investigated. In this chapter, I calculate stamen natural frequency in six *Solanum* taxa, which vary in their stamen morphology and mating system: three are highly heterantherous and outcrossing, and three are weakly heterantherous and selfing. Based on a cantilever model of the stamen, I measure its displacement under known weights and calculate its flexural rigidity and natural frequency.

I ask the following questions:

1. How does the natural frequency of stamens vary between contrasting anther types?
2. How does the natural frequency of each type of stamen vary between different mating systems?
3. How do these measurements of natural frequency compare to those measured using laser Doppler vibrometry?

Chapter four: Bumblebee buzzing behaviour in two morphologically distinct Solanum species

Floral vibrations are costly for bees to produce, but matching their strategies to different floral morphologies may allow them to reduce these costs (Raine and Chittka, 2007; Switzer et al., 2019). Bees may benefit from exploiting the biomechanical properties of flowers by matching their buzz frequency to stamen natural frequency, thus amplifying their vibrations, and increasing pollen release (Rosi-Denadai et al., 2020). We might expect bees employing frequency-matching to adjust their buzz characteristics to differences in stamen natural frequency between buzz-pollinated species (Nunes et al., 2021). In this chapter, I investigate the effects of plant species and handling experience on the frequency and duration of bumblebee floral vibrations. I assess buzzes by individually marked foragers on multiple visits to two species which differ in stamen natural frequency and pollen release rates: the large-flowered, highly heterantherous *Solanum citrullifolium*, and the small-flowered, weakly heterantherous *S. heterodoxum*.

I ask the following questions:

1. How does bumblebee body size affect buzz characteristics in floral vibrations and flight buzzes?
2. Do bumblebee buzzes change in frequency or duration between plant species?
3. Do buzzes change within single visits to either species?
4. Do buzzes change with experience across multiple visits to either species?

Chapter five: Floral orientation affects pollen deposition in buzz-pollinated flowers with bilateral symmetry

Floral orientation plays a key role in plant-pollinator interactions and is associated with floral symmetry (Giurfa et al., 1999; Fenster et al., 2009). Bilaterally symmetrical flowers are often oriented horizontally for optimal pollinator positioning and pollen transfer efficiency, whilst there can be more variability in the orientation of radially symmetrical flowers (Armbruster and Muchhala, 2020). Buzz-pollinated flowers display a range of morphologies and orientations, including bilateral, horizontally oriented (for example, heterantherous) flowers and radial, pendant (for example, cone) flowers (Bohs et al., 2007). The effect of orientation on pollen transfer is likely to differ between radial, cone flowers and heterantherous, bilateral flowers. In this chapter, I examine the effect of floral orientation on bumblebee-mediated pollen deposition in three *Solanum* species with different natural orientations: *S. lycopersicum* and *S. seaforthianum* (radial, pendant), and *S. rostratum* (bilateral, heterantherous, horizontal).

I ask the following questions:

1. Does orientation (pendant vs horizontal) affect total pollen deposition (self and outcross) in these three species?
2. Does donor or recipient orientation affect outcross pollen deposition in *S. rostratum*?

3. Does the correspondence between donor and recipient orientation affect outcross pollen deposition in *S. rostratum*?

Chapter six: General discussion

I discuss my findings within the context of previous work on three key aspects of buzz pollination:

1. The role of stamen arrangement in buzz pollination, with reference to three broad types of flowers.
2. The division of labour within heterantherous buzz-pollinated flowers.
3. The effect of floral morphology on bee foraging behaviour.

Chapter Two: Transmission of bee-like vibrations in buzz-pollinated plants with different stamen architectures

This chapter has been published as:

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2.1 Abstract

In buzz-pollinated plants, bees apply thoracic vibrations to the flower, causing pollen release from anthers, often through apical pores. Bees grasp one or more anthers with their mandibles, and vibrations are transmitted to this focal anther(s), adjacent anthers, and the whole flower. Pollen release depends on anther vibration, and thus it should be affected by vibration transmission through flowers with distinct morphologies, as found among buzz-pollinated taxa. We compare vibration transmission between focal and non-focal anthers in four species with contrasting stamen architectures: *Cyclamen persicum*, *Exacum affine, Solanum dulcamara* and *S. houstonii*. We used a mechanical transducer to apply bee-like vibrations to focal anthers, measuring the vibration frequency and displacement amplitude at focal and non-focal anther tips simultaneously using high-speed video analysis (6,000 frames per second). In flowers in which anthers are tightly arranged (*C. persicum* and *S. dulcamara*), vibrations in focal and non-focal anthers are indistinguishable in both frequency and displacement amplitude. In contrast, flowers with loosely arranged anthers (*E. affine*) including those with differentiated stamens (heterantherous *S. houstonii*), show the same frequency but higher displacement amplitude in non-focal anthers compared to focal anthers. We suggest that stamen architecture modulates vibration transmission, potentially affecting pollen release and bee behaviour.

2.2 Introduction

Insects use substrate-borne vibrations in a range of ecological contexts, including conspecific communication and the detection of prey and predators (Cocroft and Rodríguez, 2005; Mortimer, 2017). These vibrations are often produced and detected on plant material, and the physical properties of the plant substrate, such as stem stiffness or leaf thickness, often affect vibration propagation (Cocroft et al., 2006; Kollasch et al., 2020; Velilla et al., 2020). Approximately 6-8% of angiosperms are buzz-pollinated, relying on substrate-borne vibrations (floral buzzing), typically produced by bees, to release pollen from flowers with specialised morphologies (Buchmann, 1983; Vallejo-Marín, 2019). While buzz pollination is a widespread plant-insect interaction common in agricultural and natural ecosystems, its biomechanical aspects remain understudied compared to other insect vibrations, such as those used in communication.

More than half of all bee species can buzz to collect pollen, and the behaviour is thought to have evolved approximately 45 times within bees (Anthophila) (Cardinal et al., 2018). During buzz pollination, the bee typically clutches the anthers with its mandibles and produces thoracic vibrations using the indirect flight muscles (Buchmann, 1983; King and Buchmann, 2003). These vibrations are transmitted to the flower, triggering pollen release. Most buzz-pollinated flowers have tubular anthers that dehisce only via small apical pores or slits, i.e., poricidal anthers, through which small, dry pollen grains are released during floral vibrations (Harder and Barclay, 1994). Moreover, some species with longitudinally dehiscent anthers have evolved floral morphologies which also rely on floral buzzing for pollen release. For example, the modified corolla of some *Pedicularis* species encloses the anthers in a tube, which thus functions analogously to an individual poricidal anther (Corbet and Huang, 2014). Furthermore, many species with non-poricidal anthers and apparently accessible pollen, e.g., *Rosa* or *Begonia* species, are often buzzed by bees, presumably maximizing pollen collection (Buchmann, 1985; Russell et al., 2020). The interaction between flower and vibrating bee is thus very widespread, emphasising the importance of studying floral vibrations in detail across plant lineages.

Similar to the study of vibrations used for insect communication, the functional study of floral vibrations can be divided into three major stages: (1) the production of vibrations by the bee, (2) the propagation of these vibrations through the bee-flower coupled system, and (3) the effect of vibrations on pollen release (Oberst et al., 2019; Vallejo-Marín, 2019). Most work to date has focused on (1) bee buzzing behaviour and/or (3) pollen release. Bees produce floral vibrations which vary in duration, frequency (oscillations per unit time) and amplitude, the primary components with which vibrations can be described (Sueur, 2018; Vallejo-Marín, 2019). Vibration amplitude, whether measured as displacement, velocity, or acceleration, has a significant and positive effect on pollen release: higher amplitude vibrations release more pollen (De Luca et al., 2013; Rosi-Denadai et al., 2020; Kemp and Vallejo-Marín, 2021). In contrast, the effect of vibration frequency on pollen release appears to be weaker within the natural range of bee buzzes (~100 - 400 Hz; De Luca and Vallejo-Marín, 2013; De Luca et al., 2019; Rosi-Denadai et al., 2020), although vibrations at much higher frequencies than those produced by bees do result in the release of more pollen (Harder and Barclay, 1994; Arceo-Gómez et al., 2011).

Buzz-pollinated flowers are morphologically diverse, yet the intra-floral transmission of vibrations across a range of species has been rarely investigated (but see the floral manipulation experiment by (Bochorny et al., 2021). The structure of the androecium, e.g., the spatial arrangement of the anthers, is likely to affect the transmission of vibrations. Here we follow Endress (1996) and define stamen architecture as the relative sizes of stamens, their degree of fusion, and their spatial and functional connections (Jeiter et al., 2020). Many taxa with poricidal anthers have converged on a stamen architecture in which equally sized anthers are held tightly together forming an anther cone as in *Solanum dulcamara* L. and *S. lycopersicum* L. (Glover et al., 2004). Interestingly, this anther cone morphology has evolved independently in many other groups of flowering plants (Faegri, 1986; Glover et al., 2004; Vallejo-Marín et al., 2010). During floral buzzing, bees often use their mandibles to hold only a subset of the anthers in the flower (Papaj et al., 2017). In species with tightly arranged anthers, bee vibrations applied to one or a few anthers are likely to be effectively transmitted to the rest of the anther cone. In contrast, in buzz-pollinated species with anthers presented more loosely (e.g., most *Melastomataceae*, *Solanum elaeagnifolium* Cav., *S. sisymbriifolium* Lam.), applying vibrations to a subset of focal anthers might limit transmission to non-focal anthers in the same flower.

This potential difference in vibration transmission between focal and non-focal anthers is perhaps best exemplified in heterantherous species, in which two or more morphologically distinct sets of anthers occur in the same flower (Vallejo-Marín et al., 2010). In some heterantherous species, the two anther sets perform different functions, with long anthers contributing disproportionately to pollination (pollinating anthers) and short anthers (feeding anthers) being the focus of attention of buzz-pollinating bees (Müller, 1881; Luo et al., 2008; Vallejo-Marin et al., 2009). A recent study has shown that pollinating and feeding anthers of heterantherous *Solanum* differ in natural frequency also known as the first mode of vibration, which is the lowest frequency at which a material object vibrates when disturbed. This difference is likely a result of differences in biomechanical properties including size and shape (Nunes et al., 2021). Despite the potential for differences in anther and floral characteristics, such as those described above, to affect the transmission of vibrations in buzz-pollinated flowers, few studies have explicitly compared floral vibrations across different floral morphologies (Bochorny et al., 2021).

Here, we used a mechanical transducer to apply bee-like artificial vibrations to focal anthers, simultaneously measuring the vibration frequency and displacement amplitude at the tips of focal and non-focal anthers of the same flower in two axes using high-speed video analysis (6,000 frames per second). We used four buzz-pollinated species with contrasting floral and poricidal anther morphologies: *Cyclamen persicum* Mill. (Primulaceae)*, Exacum affine* Balf. ex Regel (Gentianaceae)*, Solanum dulcamara* and *S. houstonii* Dunal (Solanaceae). The arrangement of anthers within these flowers varies from a tight cone (*S. dulcamara*) to a loose, heterantherous assemblage (*S. houstonii*). We ask the following questions: i) Does the dominant frequency of vibrations change between focal and non-focal anthers in these flowers? ii) Does vibration amplitude (measured as displacement amplitude) change between focal and non-focal anthers? iii) Do vibration characteristics depend on plant species and/or the characteristics of the applied vibration? Based on previous work suggesting conservation of frequency properties during buzz pollination (De Luca et al., 2018; Pritchard and Vallejo-Marín, 2020), but changes in amplitude as vibrations travel through the flower (King and Buchmann, 1996; Arroyo-Correa et al., 2019), we predict that vibration amplitude, but not frequency, will be transmitted from focal to non-focal anthers less faithfully in flowers with looser anther arrangements. High-speed video requires no physical interference with the system and is an alternative to other non-contact methods to study vibrations across complex structures such as laser scanners. Our study allows us to quantify and compare the transmission of floral vibrations in flowers with different types of stamen architectures and may be useful for future work on the function and evolution of different floral morphologies among buzz-pollinated plants.

2.3 Methods

2.3.1 Study system and plant material

We studied flowers of four species from three families: *Cyclamen persicum* Mill. (Primulaceae), *Exacum affine* Balf.f. ex Regel (Gentianaceae), *Solanum dulcamara* L. and *S. houstonii* Martyn (Solanaceae). These species have contrasting stamen architectures and are nectarless, offering only pollen as a reward to floral visitors (Figure 2.1B-E). *Cyclamen persicum* flowers have poricidal anthers fused by the filaments (connate) into a symmetrical conical shape (Schwartz-Tzachor et al., 2006). Together with other *Cyclamen* species, *C. persicum* was historically presumed to be buzz-pollinated, based on the presence of poricidal anthers. However, buzz-pollinating visitors are rarely observed in wild populations and its main pollinators are often moths, hoverflies and small bees (Schwartz-Tzachor et al., 2006). *Exacum affine* has unfused (distinct), slightly curved, poricidal anthers and is primarily buzz-pollinated (Endress, 2012; Russell et al., 2015). *Solanum dulcamara* has poricidal anthers, which are fused into a single cone (connivent), with very short filaments (Glover et al., 2004). *Solanum houstonii* has unfused (distinct), curved, poricidal anthers and is heterantherous: it has two short anthers (feeding anthers) presumed to be mainly involved in attracting and rewarding pollinators, and three longer, S-shaped anthers (pollinating anthers) presumed to contribute disproportionally to pollination (Papaj et al., 2017). Both *Solanum* species are visited and buzz-pollinated by bees of diverse sizes and morphologies, although the most effective pollination of *S. houstonii* is presumably performed by relatively large bees, such as *Bombus sp., Centris sp.* and *Xylocopa sp.* (Macior, 1964; Free, 1970; Glover et al., 2004; Papaj et al., 2017; Carbonell, 2019), (L. N. pers. obs.).

Plants were purchased as full-grown plants or grown from seeds or cuttings in university greenhouses in Tucson, AZ and Pittsburgh, PA. *Cyclamen persicum* plants were sourced from Lowe’s Home Improvement. *Exacum affine* plants of three varieties

(Champion Blue, Royal Blue, Little Champ Blue) were sourced from the wholesaler Fred C. Gloeckner & Co. *Solanum dulcamara* cuttings were collected from wild populations in Pittsburgh, PA. *Solanum houstonii* seeds were sourced from the Sonoran Desert Museum, Tucson, AZ; originally collected from wild populations in Mexico.

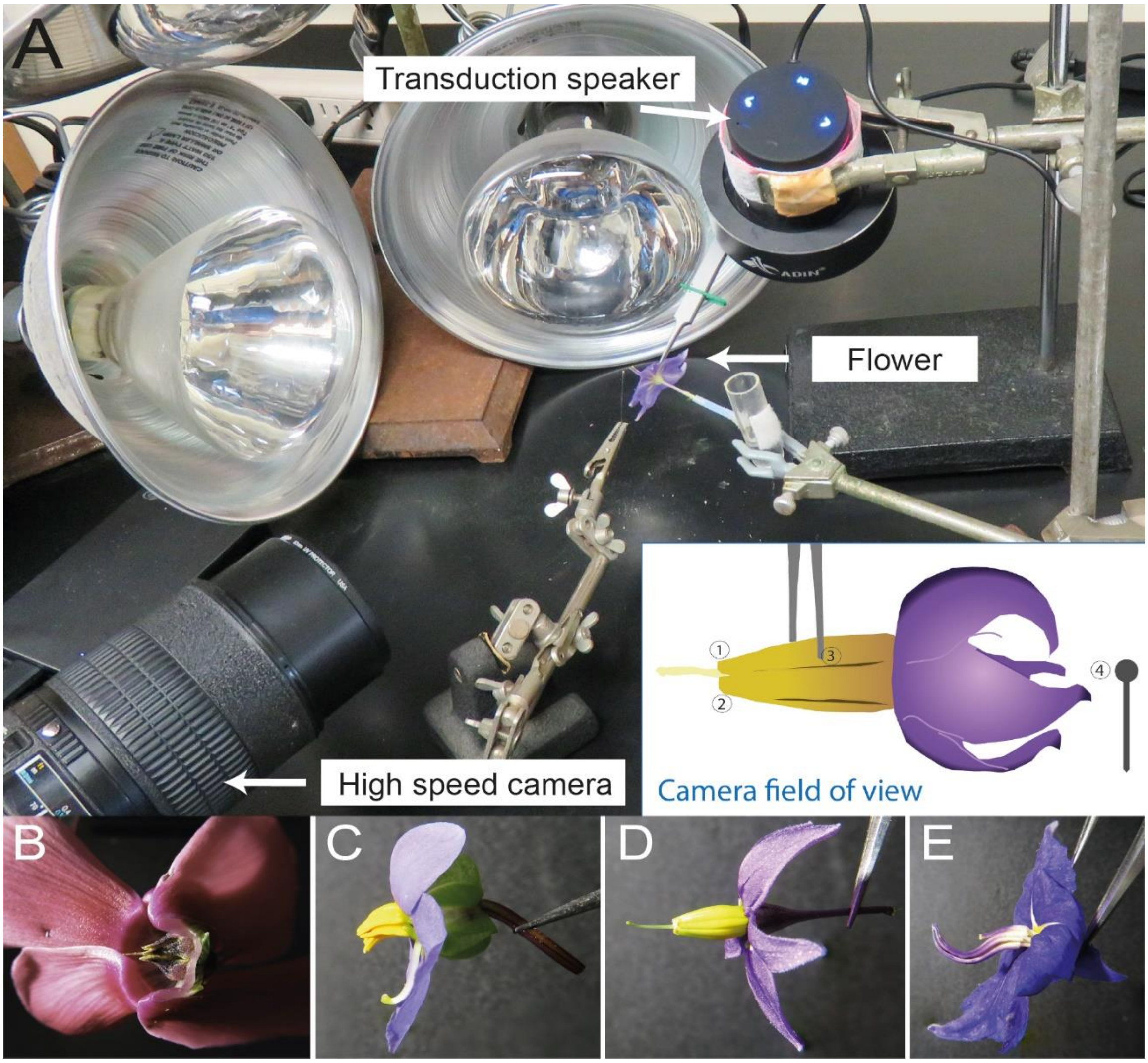

Figure 2.1 Experiment set up of the artificial vibration playback system. The inset shows a diagram of the camera field of view. 1: Focal anther tip; 2: non-focal anther tip; 3: forceps tip; 4: insect pin for calibration. Lateral view of the flowers of the four species studied here: B: *Cyclamen persicum*. C: *Exacum affine*. D: *Solanum dulcamara*. E: *Solanum houstonii*. Plant photos provided by Avery Russell.

2.3.2 Synthesis and playback of vibration signals

The experiment consisted of generating synthetic vibrations and applying them to individual flowers using a vibration transducer mechanical system, to analyse the vibrational properties of different parts of the flower (Figure 2.1A). Flowers used in experiments were as fresh as possible, usually newly opened on the plant each morning of the experiment. Synthetic vibrations consisted of 1 s pure tone signals of fixed amplitude with a 10 ms fade in and 10ms fade out. We conducted two sets of experiments. In the first set, we varied relative amplitude of the signal, while keeping frequency constant. Signals were generated by creating a sine wave with frequency of 350 Hz, using the Tone function in Audacity ver. 2.1.0 (http://audacityteam.org/) and saved as a single-channel audio file (WAV) at 44.1 kHz sampling rate. We used four relative amplitude levels: (in dB): -15, -10, -5 and 0. The absolute displacement amplitude of the vibrations applied to the flower in each of these treatments was calculated using the observed displacement of the forceps tips (see *Digitising Video Files and Time Series Analysis* section). For each amplitude, we conducted 2-3 vibration playback replicates per species in each of two species selected based on flower availability (*Exacum affine* and *Solanum houstonii*). Overall, in this experiment, we used 18 flowers, 9 of each species. In the second experiment, we generated signals as above with constant relative amplitude (0dB) but with different individual frequencies (150, 200, 250, 300, 350, 400, 450, and 500 Hz). The frequency values we used reflect the range of frequencies recorded from bees vibrating on buzz-pollinated flowers (De Luca and Vallejo-Marín, 2013; De Luca et al., 2019; Vallejo-Marín and Vallejo, 2021). For each frequency, we conducted 3-4 playback replicates for each of the four species studied, depending on flower availability. Overall, in this experiment, we used 104 flowers, 26 of each of the four species.

We played back each vibration signal using a Zoom H2 audio recorder (Zoom Corporation; Tokyo, Japan) connected to a vibration speaker (Adin S8BT 26W). The output volume of the Zoom H2 and vibration speaker was kept constant, except as noted in the Results section. The vibration speaker was modified as described in Brito et al. (2020) to transduce the vibrations to the flower by fixing a metal rod to the vibrating plate of the speaker and attaching a pair of very fine tipped forceps (Fine Science Tools, Dumont #5 Biology Tip Inox Forceps) to the end of the rod. The forceps were used to hold 1-2 anthers (the short anthers in the case of *Solanum houstonii*) (see Figure 2.1A for setup). Individual flowers were placed in floral water tubes, with the stamen's long axis parallel to the ground, i.e., flowers were kept horizontal to the ground as they would be perceived by a pollinator directly approaching the centre of the flower. The movement of the forceps was thus perpendicular to the anthers. The forceps were clamped at approximately the same position (1/4 of the anther length from the connection with the filament) on the anthers for each trial. In trials on *C. persicum*, one petal was cut away to allow visualisation of the anthers. A fresh flower was used for each replicate such that each flower was vibrated only once, and we collected data sequentially for each amplitude level or frequency before moving to the next set of replicates to control for effects of time of day on vibration characteristics.

2.3.3 High speed digital imaging

To analyse the vibration of different parts of the flower simultaneously, we used high-speed digital imaging, which allowed us to simultaneously track the movement of captured objects along two dimensions at different locations of the image frame. We recorded the vibrating flowers at 6,000 frames per second (fps; 1280 x 512 pixels) against a black background using a FASTCAM SA-8 camera (Photron, San Diego, California USA) and halogen bulbs for illumination. Recording started before the vibration playback began and captured the whole 1 second vibration. As there was some slight variation in the distance between the camera and the flower among replicates, an entomological pin of known size (size 1) was kept in shot for videos, to enable size calibration. We used the pin's width in mm to calibrate the displacement in mm for the output, allowing consistency in displacement measurement across different videos (Figure 2.1A).

2.3.4 Digitising video files and time-series analysis

All video footage was analysed in two dimensions using the *DLTdv7* digitising tool (Hedrick, 2008) in MATLAB 9.6 (R2019a; MathWorks Inc). Recordings were 730 ms long on average. This digitising tool allows point tracking in high-speed video footage (Varennes et al., 2019), and we used it to generate a time series of *x*-*y* coordinates for each tracked point. For each video, we simultaneously tracked three points through time to extract vibrational information measured as displacement: (1) The tip of the forceps, hereafter *control*. This allowed us to empirically obtain frequency and displacement amplitude of the input vibrations transduced to the flowers, and to account for variation in volume playback introduced during the experiment. (2) The tip of the anther held by the forceps, hereafter the *focal anther*. (3) The tip of the anther furthest away from the focal anther, hereafter the *non-focal anther*. In a few cases, it was not possible to track all three points for each sample due to obstruction of the control point by other parts of the flower or due to low light. All three points were reliably tracked in 87 out of 122 samples.

The x-y time series data were analysed using the *seewave* package (Sueur et al., 2008) in R ver. 4.0.2 (R Core Development Team, 2020). Displacement values (calibrated to mm, using the insect pin described previously as a reference for size) were calculated for x- and y-axes, by zero-centring the data. These x-y displacements were used to obtain an overall measure of displacement magnitude defined as (x-displacement^2 + y-displacement^2)$^{1/2}$. We used a high pass filter of 80Hz using the *fir* function (Hanning window, window length = 512 samples). For each digitised recording, a section of 100 ms in the middle of each time series was selected, where the vibration was more stable (approximately from 0.3 seconds to 0.4 seconds for every sample). Twelve digitised samples which were too short were removed from the dataset, leaving 75 samples remaining as the final total sample size. From these 100 ms sections (sampled at 44,100 samples per second), we computed the frequency spectra using the function *spec* (using power spectral density) and calculated the dominant frequency using the function *fpeaks* (nmax =1). We also estimated peak displacement amplitude (D_P), peak-to-peak displacement (D_{P-P}), and Root Mean Squared (D_{RMS}) displacement using the functions *max* (on absolute values), *max – min*, and *rms*, respectively. These are commonly used parameters describing vibration properties in buzz pollination (Vallejo-Marín, 2019). For example, the dominant frequency is the frequency of the sinusoidal component with the highest relative amplitude, while D_{RMS} reflects the overall energy content of a vibration (Sueur, 2018).

2.3.5 Statistical analysis

We evaluated the correlation between the different measurements of displacement amplitude (D_P, D_{P-P}, D_{RMS}), and between displacement in the x-, y-axis and v-vector using Pearson moment correlations. We assessed the association between the characteristics of the input vibrations applied by the forceps (dominant frequency and D_{RMS}) and those measured at the anther tips using linear models fitted with the function *lm*. In these models, vibration dominant frequency or D_{RMS} were used as the response variable, and input vibration (at the forceps tip), anther type (non-focal and focal anthers) and species as the explanatory variables. For each model, diagnostics were produced using the package DHARMa (Hartig, 2019). For those which showed significant outliers, models were re-created without these data points to assess whether any effects were driven by these extreme observations. The statistical significance of effects remained similar and therefore we kept the full data set for the final analysis. Statistical significance of the main effects and their interactions were assessed using Type III sums of squares using the package *car* (Weisberg and Fox, 2011). Model predictions were plotted using *plot_model* (type=pred) in the package *sjPlot* (Ludecke, 2021). All statistical analysis was performed in R 4.0.2 (R Development Core Team, 2021).

2.4 Results

2.4.1 Frequency of anther vibrations

The dominant frequencies measured in the x- and y-axis were highly correlated across all samples. (Pearson's correlation *r:* 0.98, df: 266, $p < 0.001$) (Figure 2.2A). Dominant frequency across anthers and plant species ranged from 150 to 529Hz (Figure 2.7). Forceps dominant frequency was the only significant predictor of anther dominant frequency in our linear model ($p < 0.001$, Table 2.1A), and we found no effect of either anther type or plant species (i.e., anther arrangement) on the dominant frequency of vibrations measured at the tips of anthers ($p > 0.05$, Table 2.1A). In other words, the dominant frequency did not change as vibrations were transmitted through the flowers from the forceps. The overall frequency spectra were also similar between species and anther types, with very few harmonics in any of the vibrations (Figure 2.3).

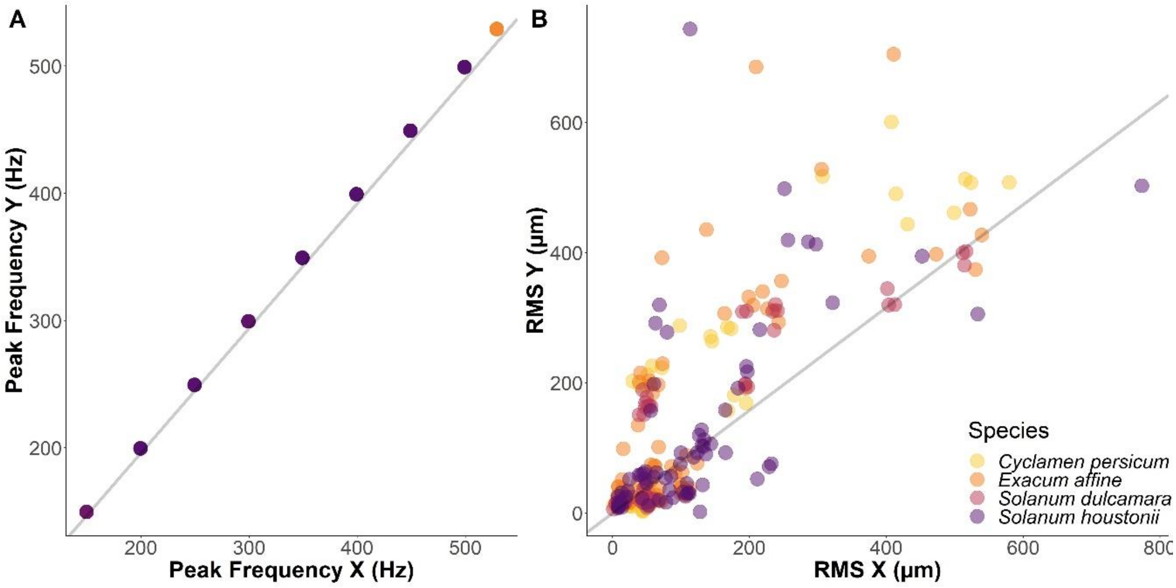

Figure 2.2 Measurement of (**A**) frequency (dominant frequency, Hz) and (**B**) root mean square amplitude (D_{RMS}, μm) in either the x- or y-axis. Grey lines indicate 1:1 relationship.

	Estimate	**Std. error**	***P*-value***	***P*-value****
A. Dominant frequency (Hz)				
Forceps dominant frequency	1.000e+00	1.552e-16	**<0.001**	
Anther (non-focal)	3.459e-14	3.077e-14	0.263	
Species				
(*Exacum affine*)	6.634e-14	4.279e-14	0.123	
(*Solanum dulcamara*)	7.741e-14	4.711e-14	0.103	
(*Solanum houstonii*)	6.576e-14	4.446e-14	0.141	
B. Displacement amplitude D_{RMS} (μm)				
Forceps D_{RMS}	1.012	0.045	**<0.001**	**<0.001**
Anther (Non-focal)	-16.825	9.464	0.078	0.078
Species				0.13
(*Exacum affine*)	-2.68	7.917	0.735	
(*Solanum dulcamara*)	-2.969	8.775	0.736	
(*Solanum houstonii*)	12.441	8.05	0.124	
Forceps D_{RMS}: Anther (non-focal)	0.202	0.064	**0.002**	**0.002**
Anther (non-focal): Species				**<0.001**
Anther: (*Exacum affine*)	44.754	11.306	**<0.001**	
Anther: (*Solanum dulcamara*)	1.31	12.303	0.915	
Anther: (*Solanum houstonii*)	40.533	11.792	**<0.001**	

Table 2.1 Parameter estimates of the linear models fitted with either dominant frequency (Hz) or DRMS as response, and forceps dominant frequency or DRMS, anther type, and species as explanatory variables. *P-value of explanatory variable in linear model. **P-value calculated using Type III sums of squares. Sample size is 150 for both models.

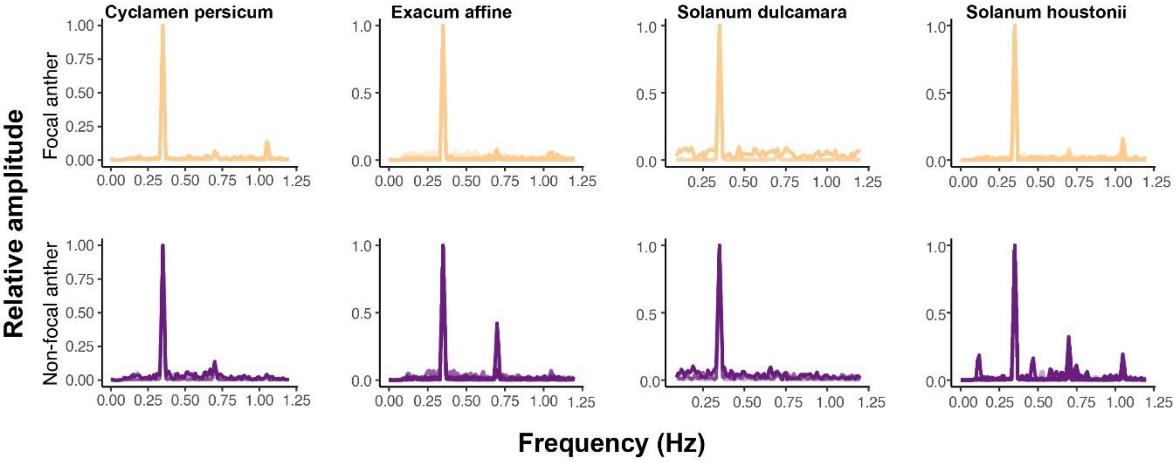

Figure 2.3 Frequency spectra. Focal (top row) and non-focal anthers (bottom row) of four plant species in response to artificial vibrations applied in the focal anther using the vibration playback system shown in Figure 2.1. The frequency of the input vibration was 350 Hz.

2.4.2 Amplitude of anther vibrations

All three measures of displacement amplitude differed slightly between the x- and y-axis across all samples, including in the forceps (Table 2.2). The average amplitude was higher in the y-axis, particularly in D_P and D_{P-P} (Table 2.2). Axes were nonetheless strongly correlated for all measures of amplitude: D_P (r: 0.81, df: 266, $p < 0.001$); D_{P-P} (r: 0.82, df: 266, $p < 0.001$); D_{RMS} (r: 0.79, df: 266, $p < 0.001$) (Figure 2.2b for D_{RMS} correlations). Therefore, we used the vector magnitude (see Methods for details) for downstream analysis on amplitude, to capture variation in displacement in both x and y axes.

We extracted three measures of displacement amplitude: D_P, D_{P-P}, and D_{RMS}. D_P across anther types and species ranged from 16.4μm to 1030μm (mean 195), D_{P-P} ranges from 39.3 to 1840 μm (mean 353), D_{RMS} ranged from 6.94 to 363μm (mean 77.8). The highest displacements for all measures were from vibrations in the non-focal anther of *S. houstonii* (heterantherous and loosely arranged stamens), and the lowest were from the non-focal anther of *C. persicum* (stamens fused in a cone). All three measures of displacements were strongly correlated across all trials: D_P and D_{P-P} (r: 1, df: 179, $p < 0.001$); D_P and D_{RMS} (r: 0.98, df: 179, $p < 0.001$); D_{P-P} and D_{RMS} (r: 0.99, df: 179, $p < 0.001$). D_{RMS} was used for all further amplitude analysis.

We found a significant interaction between anther type and input D_{RMS} (measured at the forceps) on anther displacement (vector D_{RMS}), with displacement in non-focal anthers generally increasing more rapidly with input amplitude than in focal anthers (Table 2.1B, Figure 2.4). We also found a significant interaction effect between anther type and plant species on the displacement amplitude (vector D_{RMS}) of vibrations ($p < 0.001$, Table 2.1B, Figure 2.4), with higher displacements in the non-focal anthers of *E. affine* (coefficient = 42.87) and *S. houstonii* (coefficient = 46.11) (both species have loosely arranged stamens), compared to focal anthers of *C. persicum*, which has a fused stamen cone (Table 2.1B, Figure 2.4). Separate analyses of the x- and y-axes both showed significant interactions between anther type and plant species ($p<0.005$) (Figures 2.9 and 2.10; Tables 2.4 and 2.5). When we calculated the disparity in D_{RMS} (vector) between the forceps and the anther, the mean difference across both anther types in *C. persicum* and *S. dulcamara* (both with fused stamen cones) was close to zero (Table 2.3). In contrast, the mean differences (disparity in D_{RMS} between the forceps and anther) for the non-focal anthers of *E. affine* and *S. houstonii* were 36.6µm and 55.1µm respectively, and for the focal anther of *S. houstonii* it was 13.8µm (Table 2.3).

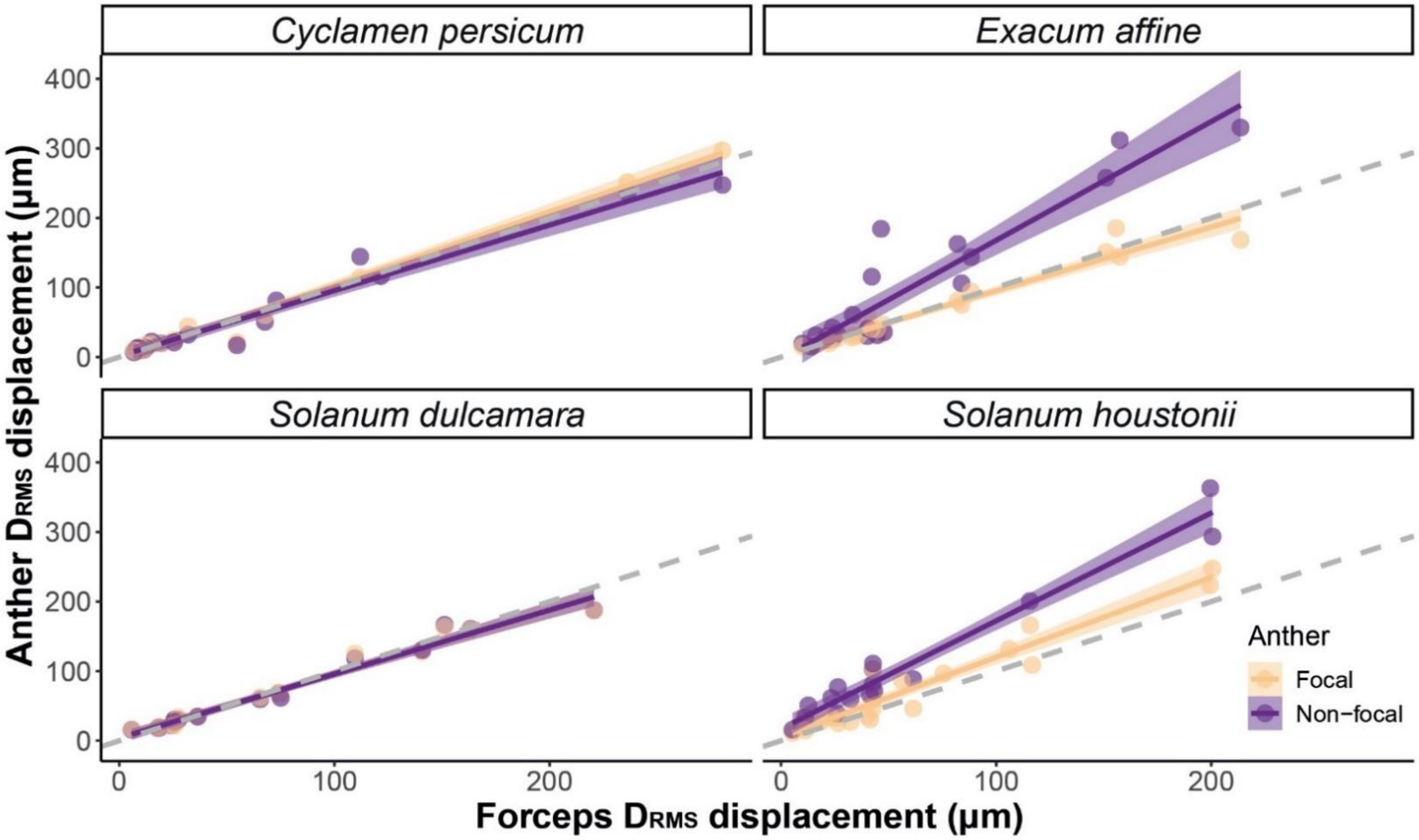

Figure 2.4 Displacement in two anther types in four species. Linear model estimates and data points for displacement (vector DRMS, µm) of focal (yellow symbols) and non-focal anther (purple symbols), against forceps displacement (vector DRMS, µm) in four plant species. Values for the vector are calculated from the x- and y-axes (see “Materials and methods” section for details). Grey dashed line indicates a linear relationship with slope=1.

Axis of measurement	X-axis		Y-axis		Vector	
Amplitude (μm)	**Range**	**Mean ± s.e.**	**Range**	**Mean ± s.e.**	**Range**	**Mean ± s.e.**
D_P	4.04 - 1500	199 ± 13.6	3.39 - 1340	230 ± 15.4	16.4 - 1030	174 ± 10.3
D_{P-P}	7.37 - 2400.7	376 ± 25.1	6.21 - 2570	437 ± 29.8	32.7 - 1840	316 ± 18.8
D_{RMS}	1.17 - 774	114 ± 8.21	1.77 - 744	130 ± 9.54	5.35 - 363	70.3 ± 4.36

Table 2.2 Summary statistics across all samples of three measures of displacement amplitude (μm) of both focal and non-focal anthers combined. The axis of measurement indicates whether the displacement was measured in the x-axis, the y-axes, or the resulting vector calculated from the combined x–y displacement (see "Methods" section). DP peak displacement amplitude, DP-P peak-to-peak displacement amplitude, DRMS root mean square displacement amplitude.

	Focal anther		Non-focal anther		
Species	**Range**	**Mean ± s.e.**	**Range**	**Mean ± s.e.**	**N**
Cyclamen persicum	-33.393 – 16.491	1.343 ± 2.8	-37.727 – 32.433	-2.476 ± 3.597	19
Exacum affine	-45.133 – 29.824	-1.312 ± 2.516	-13.069 – 154.366	36.556 ± 10.989	24
Solanum dulcamara	-33.586 – 15.332	-1.676 ± 2.699	-32.923 – 15.337	-2.017 ± 2.596	17
Solanum houstonii	-15.2 – 59.12	13.806 ± 4.079	7.286 – 174.053	55.098 ± 9.949	18

Table 2.3 Difference in displacement amplitude in μm (Anther D_{RMS} – Forceps D_{RMS}) between forceps and anther for each anther type and species across samples. Values for the vector are calculated from the x- and y-axes (see "Materials and methods" section for details). N: number of flowers.

2.5 Discussion

Our study suggests that the arrangement of poricidal anthers affects the transmission of vibrations between anthers. We found that vibrations are transmitted similarly, in both frequency and amplitude, across focal and non-focal anthers in species with stamens partially or totally fused to form a cone (*S. dulcamara* and *C. persicum*). In contrast, species in which individual stamens can move freely (*E. affine* and *S. houstonii*) showed identical frequency but higher vibration amplitudes at the tip of non-focal anthers compared to the focal anthers where vibrations were applied. Overall, the highest displacements occurred in the long anthers of the heterantherous *S. houstonii*. Our work shows that floral architecture, including the functional fusion of stamens into an anther cone, affects the transmission of vibrations applied to a subset of anthers. Because buzz-pollinating bees often grasp with their mandibles and contact with their thorax or abdomen only one or few anthers during buzz pollination, and because pollen release is a function of vibration amplitude, our results suggest that stamen architecture is an important determinant of the functional consequences of the applied vibrations.

The dominant frequency of artificial vibrations did not change as they were transmitted through flowers, regardless of flower type or vibration characteristics. This result aligns with Brito et al. (2020) who also found that artificial vibration dominant frequency is conserved throughout the heterantherous flowers of *S. rostratum*, both at anther tips and petals. Although some plant substrates such as stems can act as frequency filters (Cocroft et al., 2006), (differentially attenuating vibrations components depending on their frequency), frequency is not altered over the short distances involved in vibration transmission during buzz-pollination interactions (De Luca and Vallejo-Marín, 2013). Although the natural frequency of anthers is affected by their morphology and organisation within the flower (Nunes et al., 2021), the frequency of vibrations has limited effects on pollen release in buzz-pollinated flowers, suggesting that resonance plays a minor role within the range of frequencies produced by most bees (100 to 400Hz) (De Luca and Vallejo-Marín, 2013; Nunes et al., 2020; Rosi-Denadai et al., 2020).

In contrast, we found that the amplitudes of artificial vibrations were differentially altered as they travelled through the two types of buzz-pollinated flowers. In the flowers with more loosely arranged androecia, *E. affine* and *S. houstonii*, vibrations at the tip of the non-focal anther had generally higher displacement amplitude, i.e. moved further, than those observed in the tip of anthers being vibrated. This effect was strongest in the heterantherous *S. houstonii,* where in some cases, displacement was doubled between input and the longer, non-focal pollinating anther. In *S. rostratum,* velocity amplitude from the vibration source to the anther tips of both feeding and pollinating anthers increases up to four-fold when vibrations were applied at the base of the flower (Brito et al., 2020). Stamens can be thought of as a complex cantilever beam (a structure with one fixed end and one free end) (King, 1993). Vibration displacement amplitude at the tip of the stamen should be partly a function of the stamen's length, second moment of area, Young's modulus of elasticity, and mass (Vogel, 2013). Based on cantilever theory, we expect longer stamens to show generally higher displacements at the tip than shorter stamens. Stamen length differences may help explain the difference in vibration amplitude between the short anthers of *E. affine* and the long pollinating anthers of *S. houstonii*. However, stamen material properties, morphology and architecture are likely to affect important parameters determining their vibrational properties, including their second moment of area and Young's modulus (stiffness) (Vogel, 2013), and predictions based on length alone might not capture the behaviour of real stamens (Vogel, 2013). Previous empirical work shows that amplitude has a significant, positive effect on pollen release (Harder and Barclay, 1994; De Luca et al., 2013; Kemp and Vallejo-Marín, 2021), with increased anther acceleration causing pollen grains to gain in energy and escape through the pores at a higher rate (King and Lengoc, 1993). Clearly more work in this area is needed, including both empirical and modelling studies of the vibrational properties of stamens incorporating the complexity of the forms and material properties of stamens.

Unlike the heterogenous vibration amplitude observed between focal and non-focal anthers of species with loosely held stamens, species in which anthers are tightly held together forming tight, connivent, anther cones (*C. persicum* and *S. dulcamara*) showed vibrations of the same, uniform amplitude between focal and non-focal anthers. The functionally cohesive androecium in these species appears to homogeneously transmit vibrations across the anther cone. The uniformity of the amplitude and frequency of vibrations across all anthers of species with fully or partly fused (connate or connivent) anther cones might have implications for patterns of pollen release during buzz pollination. Species with connivent anther cones may show a more uniform rate of pollen release from each anther when vibrated, compared to the more heterogenous range of vibrations experienced by individual anthers of species in which anthers move more freely. Anther cones have evolved in a variety of taxa with buzz-pollinated flowers including species in the families, Ericaceae, Gesneriaceae, Melastomataceae, Primulaceae, Rubiaceae, and Solanaceae (Harder and Barclay, 1994; Puff et al., 1995; Glover et al., 2004; Schwartz-Tzachor et al., 2006) providing excellent opportunities to compare the functional significance of convergent floral morphologies. The same putative uniform pollen release may also occur when non-poricidal anthers are enclosed in a corolla and flowers are buzz-pollinated, as seen in some *Pedicularis* species (Corbet and Huang, 2014; Tong et al., 2019). Our study did not investigate pollen release patterns in different types of flowers and further work quantifying vibratory pollen release in flowers with disparate morphologies across taxonomic groups could help establish the functional consequences, if any, of different androecium architectures.

We suggest that the differences in vibration transmission we see in this study are largely due to differences in stamen architecture in our chosen flower types. However, other morphological differences between the four species are also likely to be important in determining vibration transmission. Studies on other types of insect vibrations have shown that flexible plant stems attenuate vibrations more than stiff stems, as do thick leaves compared with thin leaves (Cocroft et al., 2006; Velilla et al., 2020). In buzz-pollinated flowers, traits affecting vibration properties might include anther curvature (e.g. *S. houstonii*), stamen stiffness and length (Nunes et al., 2020). Similarly, the size of the anther locules (where the pollen is located before release), and thickness of the anther walls may affect vibration transmission. Few studies have examined the effect of specific morphological traits on vibration transmission in buzz-pollinated flowers, but closely-related species of *Solanum* with similar morphologies differ in their vibration transmission properties (Arroyo-Correa et al., 2019). Moreover, partial removal of stamen structures, such as the connective appendages in *Huberia bradeana* (Melastomataceae), can affect the relative amplitude of vibrations (Bochorny et al., 2021). Although the species studied here differed in anther architecture and the transmission of vibrations through the androecium, all of them have stamens positioned relatively closely together, more or less forming a cone. Other buzz-pollinated species can have stamens more widely separated and not forming a cone, such as those found in several species of Melastomataceae (Brito et al., 2016). Given the wide range of morphologies of buzz pollinated flowers (Buchmann, 1983; De Luca and Vallejo-Marín, 2013; Russell et al., 2017), we expect that a greater difference in vibration transmission could be found in species with more disparate morphologies than those studied here.

We hypothesise that differences in the transmission of vibrations observed here among species with "tight cone" vs. "loose cone" stamen architectures have functional implications for the interaction with buzz pollinators and for patterns of pollen release. If the type of stamen architecture affects vibration transmission and pollen release patterns, bee pollinators may display different behavioural strategies to buzz these flowers and maximize pollen removal, for example, by changing the manipulation of anthers during visitation. For instance, we predict that bees on flowers with loose anther arrangements might learn to simultaneously manipulate and buzz multiple anthers if this resulted in more efficient vibration transmission and thus a higher rate of pollen collection (Buchmann and Cane, 1989). In contrast, bees visiting flowers with anthers that form a tight cone may be able to extract pollen from all anthers regardless of which and how many anthers are manipulated.

From the plant perspective, the uniform vs. heterogenous transmission of vibrations from focal to non-focal anthers in species with cone vs. loose stamens could also have fitness consequences. On the one hand, efficient vibration transmission of vibrations from focal to non-focal anthers could increase pollen deposited on pollinators during single visits, potentially increasing pollen export to other flowers. On the other hand, although not observed here, vibration damping from focal to non-focal anthers could limit the amount of pollen removed from the flower during single visits and increase the release of pollen over multiple visits (pollen dispensing) (Kemp and Vallejo-Marín, 2021). The fitness consequences of these patterns of pollen release may also depend on the relative size of the interacting flower and pollinator. Bees that are small relative to the flower are often unable to buzz all anthers at once. To the extent that the visitor is too small to be a legitimate pollinator (Solís-Montero et al., 2015), reducing pollen release in non-focal anthers (for example by limiting the vibration amplitude of non-focal anthers) may limit pollen loss during visitation by floral larcenists. Both the different stamen arrangements in cone vs. loose stamens and the associated changes in floral handling by visiting pollinators might also influence the precision of pollen placement on bees' bodies (Glover et al., 2004), and thus the efficiency of pollen transfer to stigmas and the placement of pollen on "safe sites"

(Vallejo-Marin et al., 2009; Koch et al., 2017). Further studies of how vibrations are applied to flowers with different stamen architectures and their effect on pollen release, including their placement on pollinators' bodies, in both laboratory and field settings, will help ascertain the functional consequences of the enormous morphological diversity observed in buzz-pollinated flowers.

2.6 Supplementary material

X axis	Estimate	Std. error	*P*-value*	*P*-value**
Displacement amplitude D_{RMS} (μm)				
Forceps D_{RMS}	0.946	0.056	**<0.001**	**<0.001**
Anther (Non-focal)	-7.696	24.43	0.078	0.753
Species				0.846
(*Exacum affine*)	9.708	20.738	0.640	
(*Solanum dulcamara*)	7.982	22.914	0.728	
(*Solanum houstonii*)	18.722	21.106	0.887	
Forceps D_{RMS}: Anther (non-focal)	-0.037	0.082	0.652	0.652
Anther (non-focal): Species				**<0.005**
Anther: (*Exacum affine*)	29.011	29.623	0.329	
Anther: (*Solanum dulcamara*)	14.929	32.161	0.643	
Anther: (*Solanum houstonii*)	108.014	30.903	**<0.001**	

Table 2.4 Linear model for the x axis fitted with DRMS as response, and forceps DRMS, anther type and species as fixed effects. *P-value of fixed effect in linear model. **P-value calculated using Type III sums of squares. Sample size is 150.

Y axis	Estimate	Std. error	*P*-value*	*P*-value**
Displacement amplitude D_{RMS} (μm)				
Forceps D_{RMS}	1.00849	0.03535	**<0.001**	**<0.001**
Anther (Non-focal)	-23.404	16.187	0.15	0.15
Species				**<0.005**
(*Exacum affine*)	0.207	13.919	0.988	
(*Solanum dulcamara*)	-11.809	15.43	0.445	
(*Solanum houstonii*)	35.077	14.159	**<0.05**	
Forceps D_{RMS}: Anther (non-focal)	0.15	0.0514	0.004	0.004
Anther (non-focal): Species				**<0.005**
Anther: (*Exacum affine*)	100.6	19.893	**<0.001**	
Anther: (*Solanum dulcamara*)	3.928	21.628	0.856	
Anther: (*Solanum houstonii*)	11.85	20.78	0.569	

Table 2.5 Linear model for the y axis fitted with DRMS as response, and forceps DRMS, anther type and species as fixed effects. *P-value of fixed effect in linear model. **P-value calculated using Type III sums of squares. Sample size is 150.

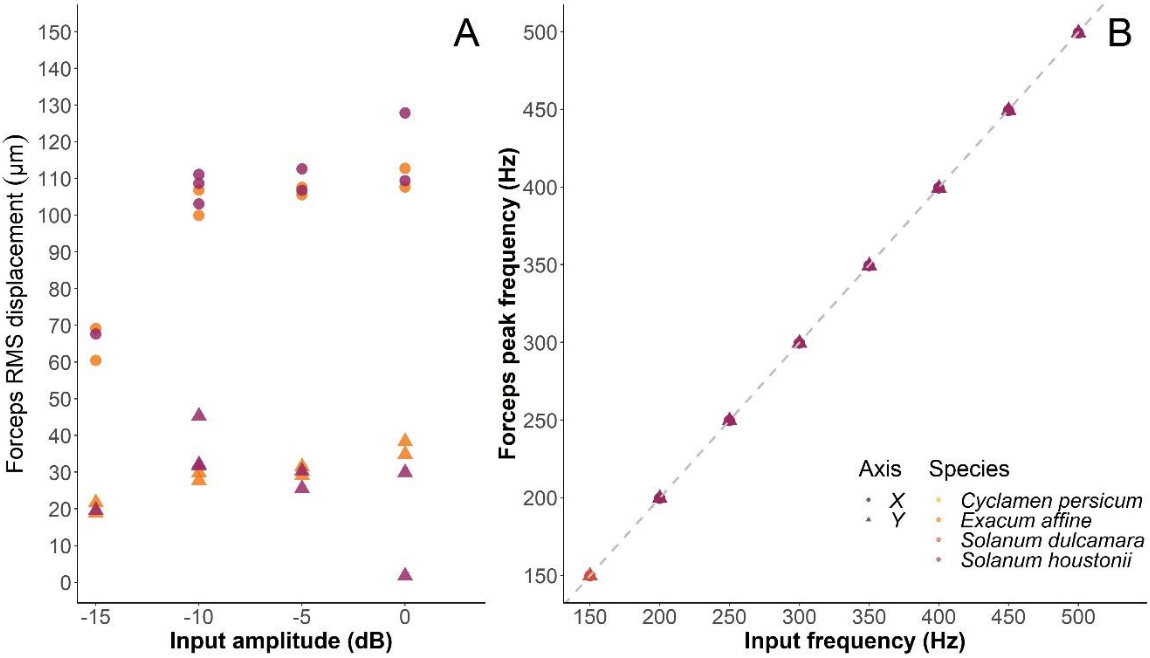

Figure 2.5 Measured RMS displacement (µm) of forceps against input amplitude (dB) (A) and measured peak frequency (Hz) of forceps against input frequency (Hz) (B). Grey dashed line indicates a linear relationship with slope=1.

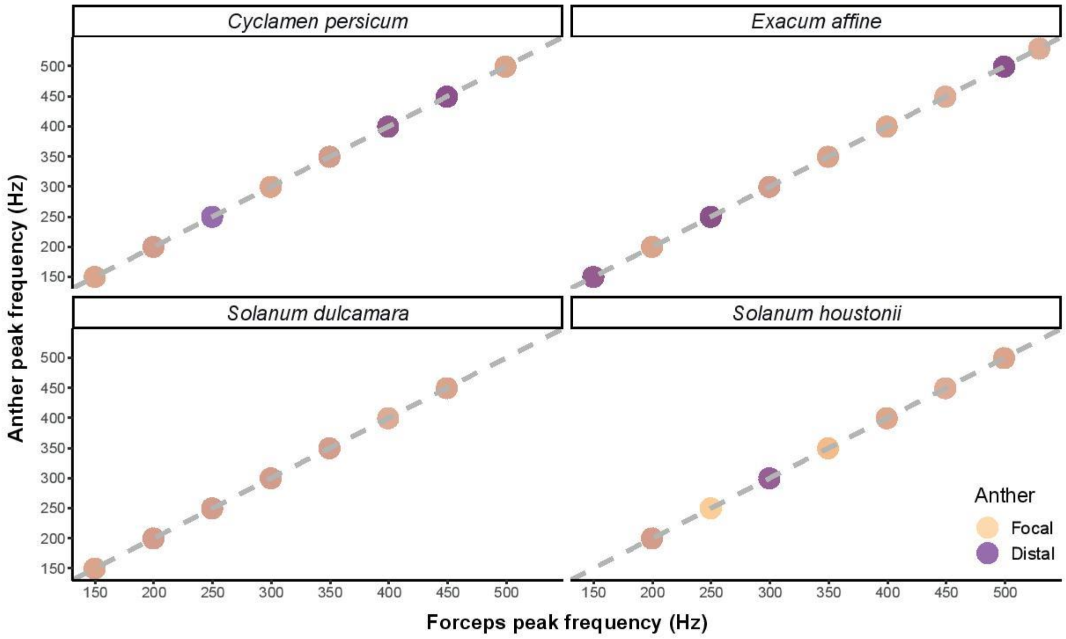

Figure 2.6 Measured peak frequency (Hz) against forceps frequency (Hz) for focal and distal anther of four plant species. Grey dashed line indicates a linear relationship with slope=1.

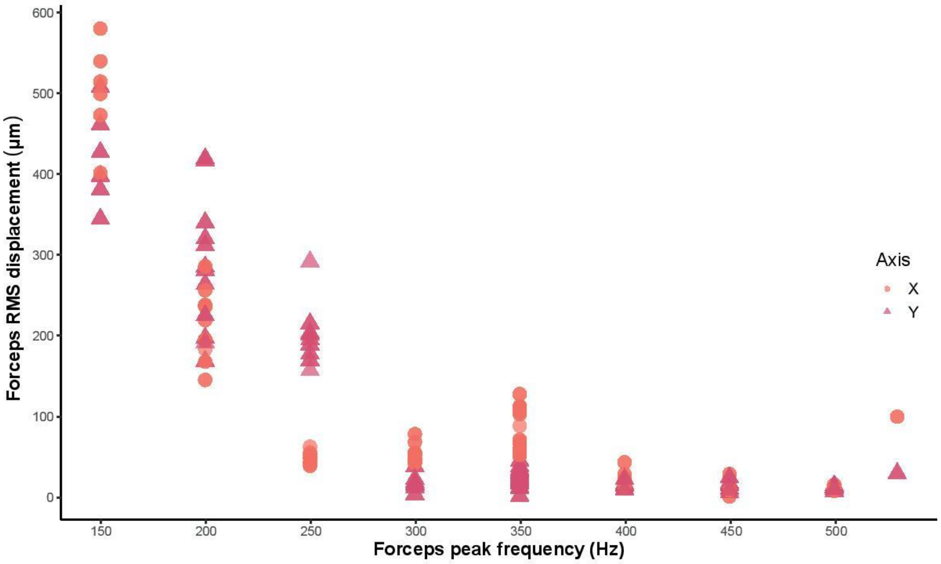

Figure 2.7 Forceps peak frequency (Hz) v forceps RMS displacement (µm) for both axes.

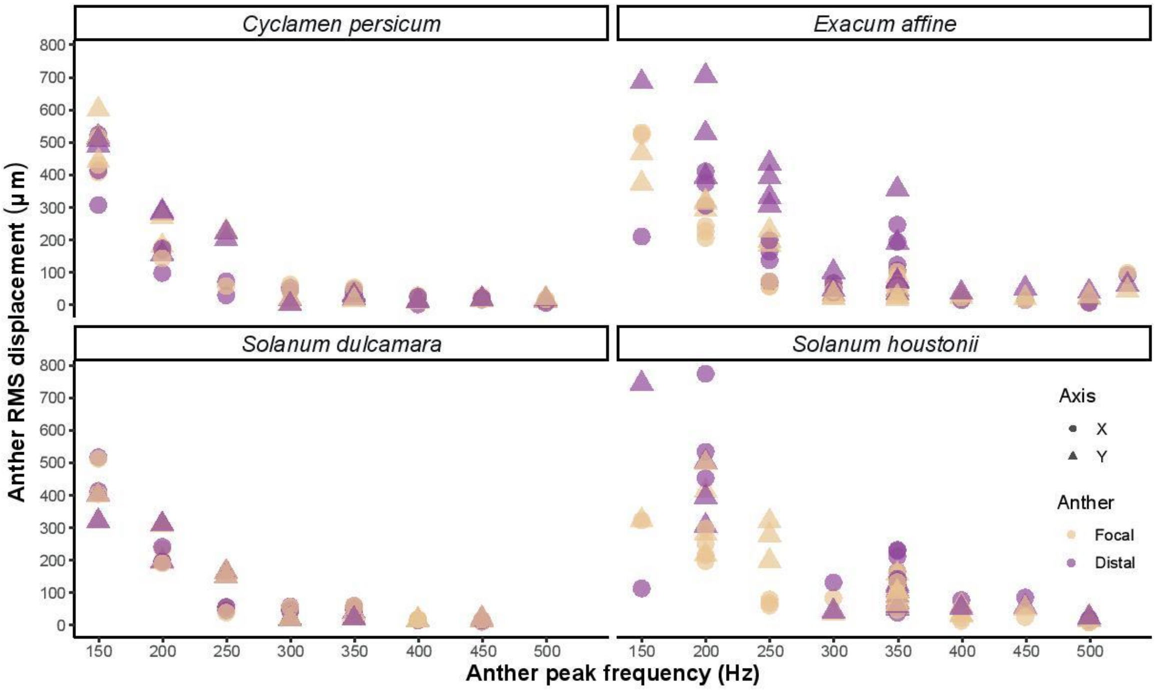

Figure 2.8 Anther peak frequency v anther RMS displacement (µm) for both axes.

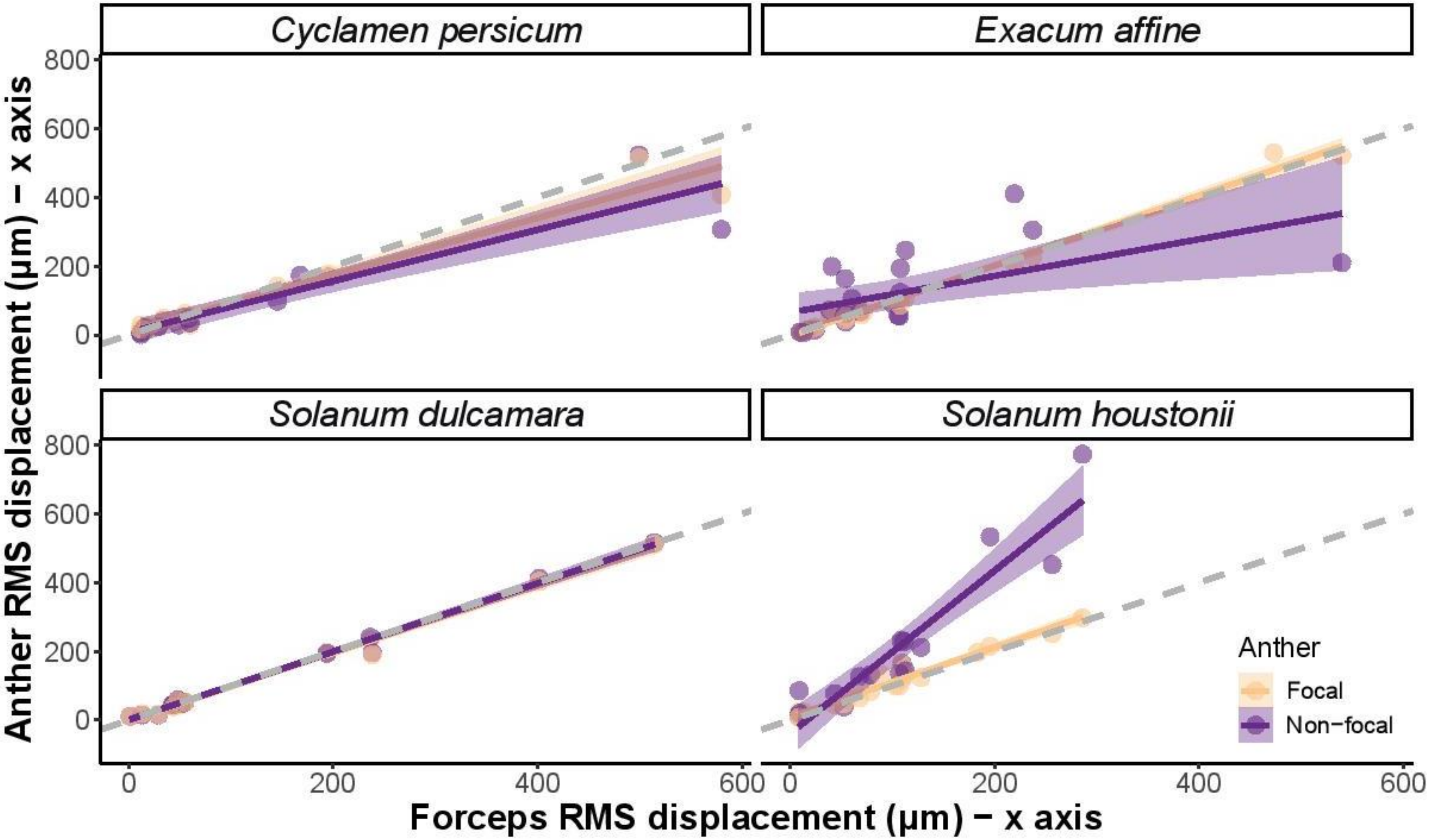

Figure 2.9 Linear model estimates and data points for measured x-axis RMS displacement (µm) of focal and distal anther against forceps RMS displacement (µm) in four plant species. Grey dashed line indicates a linear relationship with slope=1.

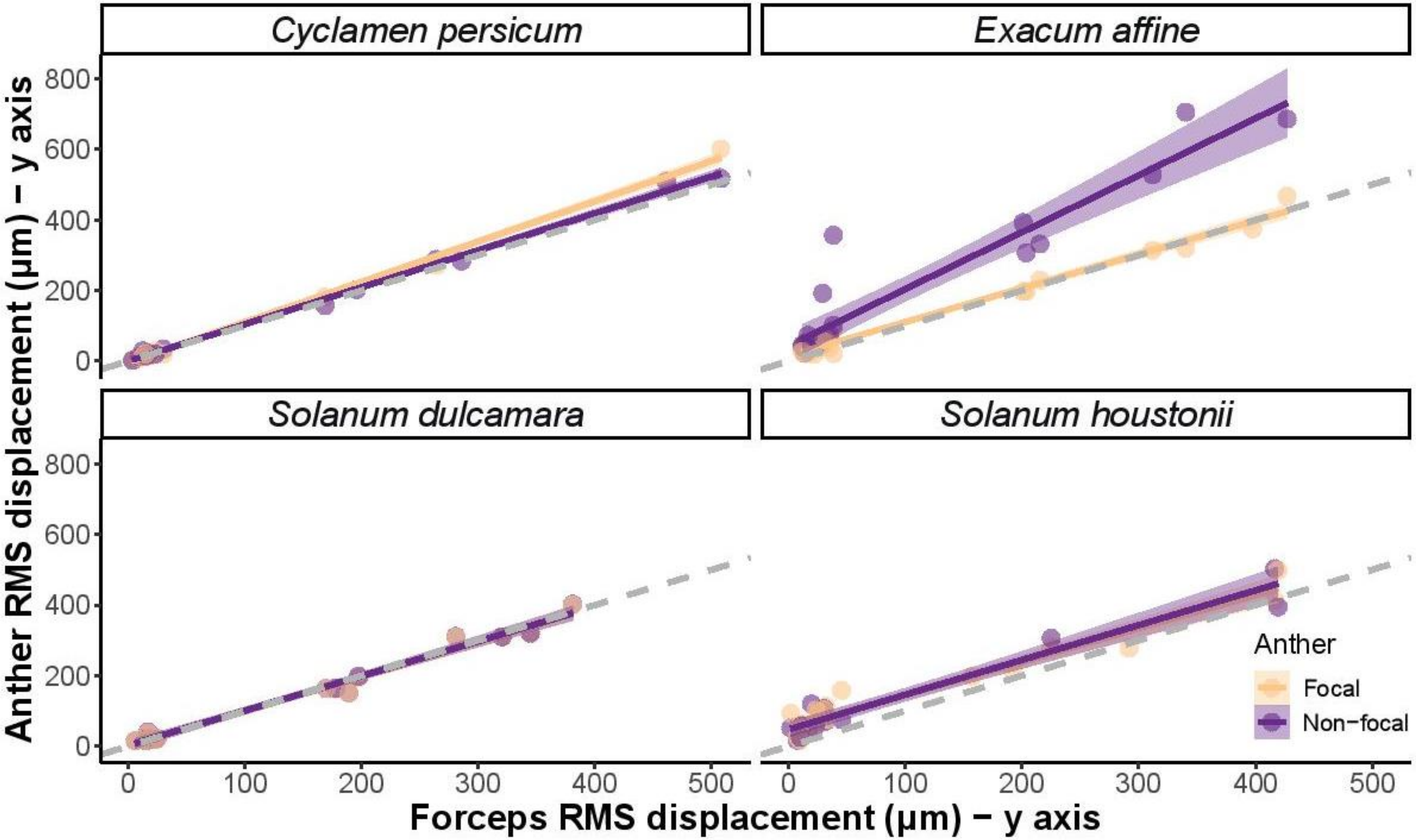

Figure 2.10 Linear model estimates and data points for measured y-axis RMS displacement (μm) of focal and distal anther against forceps RMS displacement (μm) in four plant species. Grey dashed line indicates a linear relationship with slope=1.

Chapter Three: Stamen natural frequency in six *Solanum* taxa with differing floral morphologies

Part of this chapter (floral morphological traits) has been published in:

Nunes, C. E. P., L. Nevard, F. Montealegre-Z, and M. Vallejo-Marín. 2021. Variation in the natural frequency of stamens in six morphologically diverse, buzz-pollinated, heterantherous Solanum taxa and its relationship to bee vibrations. *Botanical Journal of the Linnean Society* 197: 541-553.

3.1 Abstract

Biomechanical characteristics of buzz-pollinated flowers can affect their response to external forces, including floral vibrations. Vibrating a stamen at its natural frequency amplifies the vibrations through resonance. As vibration amplitude is positively related to pollen release, bees can theoretically exploit this resonance to maximise pollen collection. Given differences in stamen morphology within and between buzz-pollinated flowers, we also expect variation in natural frequency, which may impact pollen release and bee behaviour. This potential variation has not so far been investigated. Here, I calculate stamen natural frequency in six Solanum taxa, which vary in their stamen morphology and mating system and are heterantherous, possessing two stamen types in the same flower. I measure the displacement of the stamen under known weights and calculate stamen flexural rigidity and natural frequency based on a cantilever model of the stamen. I find that natural frequencies ranged from 87 to 203Hz, overlapping with the lower bounds of known bee vibrations, and are strongly linked to stamen length, which, in turn, is related to flower size and mating system. This method over-simplifies the variation in stamen traits within these taxa but captures differences in stamen natural frequency between species.

3.2 Introduction

All living organisms have distinct biomechanical properties, which mediate their reaction to external forces. The stiffness of stems and leaves affects a plant's response to herbivores (Kollasch et al., 2020; Velilla et al., 2020), and differences in floral properties can influence the transmission of vibrations through flowers (Chapter Two) (Arroyo-Correa et al., 2019; Nevard et al., 2021). As a result of the tight relationship between vibrations and pollen release in buzz-pollinated plants, a biomechanical framework for the structure and function of poricidal stamens is crucial to buzz pollination research (Michener, 1962; Macior, 1964; Buchmann and Hurley, 1978; Buchmann, 1983; De Luca and Vallejo-Marín, 2013). The first explicit biomechanical model of the poricidal stamen was proposed by Buchmann over forty years ago (Buchmann and Hurley, 1978), and several studies in the following decade empirically assessed stamen vibrational properties in different plant taxa (Corbet et al., 1988; King and Lengoc, 1993; King and Buchmann, 1995; King and Buchmann, 1996). Yet in the intervening years, most research has focused on bee behaviour on poricidal flowers, with less attention given to the biomechanics of buzz pollination.

One of the key biomechanical properties of flowers is stamen natural frequency. Every physical structure has a natural frequency, the frequency at which it moves when disturbed by an outside force (Niklas, 1992). Natural frequency is a function of the morphology and mass of the structure, as well as its stiffness and other material properties (Niklas, 1992). Although plant structures can have multiple natural frequencies, this study focuses on the first natural frequency: the lowest frequency at which an object moves when disturbed (Niklas, 1992; Miller, 2005). Vibrating an object at its natural frequency causes resonance: an increase in the amplitude, or energy, of these vibrations (Niklas, 1992; Timerman et al., 2014).

Most detailed research into the natural frequency of stamens has been concerned with wind-pollinated plants, which release pollen when air currents cause their long, flexible stamens to vibrate (Timerman et al., 2014). Pollen release is increased by the resonance of these stamens at their natural frequency, and this natural frequency increases as pollen is released and the mass of the anther is reduced (Timerman et al., 2014; Timerman and Barrett, 2018). Timerman and Barrett (2019) demonstrate that wind-pollination favours a lower stamen natural frequency than insect-pollination in the meadow-rue *Thalictrum pubescens* and argue that natural frequency is key to understanding evolutionary transitions from animal to wind pollination (Timerman and Barrett, 2018, 2019).

Natural frequency in buzz-pollinated flowers has been less extensively studied, but King and Buchmann (1996) measured the average natural frequency in *Solanum laciniatum* stamens to be 143Hz when containing pollen, and 102Hz when empty. Similarly, *Rhododendron* stamens have slightly different natural frequencies when containing pollen than without pollen (King and Buchmann, 1995). Although *Rhododendron* flowers have poricidal anthers (King and Buchmann, 1995; Song et al., 2019), their pollen is packaged differently to other poricidal plants and they are not obligately buzz-pollinated, although bees often buzz them to increase pollen collection (Song et al., 2019). The natural frequency of their stamens may therefore be relevant to the collection of pollen by buzzing bees even in non-poricidal plants, although this has not been studied. The distinct relationships between pollen release and biomechanical properties across different pollination systems (wind pollination, buzz pollination, non-buzzing insect pollination) demonstrates the need for more detailed research into the stamen natural frequency of buzz-pollinated flowers.

Pollen release in poricidal stamens is a function of vibration properties, including amplitude, frequency and duration (Buchmann and Hurley, 1978; De Luca and Vallejo-Marín, 2013; Rosi-Denadai et al., 2020). Crucially, the amplitude of floral vibrations is positively related to pollen release, due to additional energy being transferred to the anther and pollen grains (Buchmann and Hurley, 1978; Harder and Barclay, 1994; King and Buchmann, 1996; De Luca et al., 2013; Rosi-Denadai et al., 2020; Kemp and Vallejo-Marín, 2021). Vibrations at the stamen natural frequency, which are amplified, should therefore release more pollen than vibrations at other frequencies, and it is possible that this resonance effect is exploited by bee foragers (King and Buchmann, 1996). In contrast to wind-pollinated stamens, poricidal stamens require bee-produced vibrations for effective pollen release. These vibrations can vary in both amplitude and frequency between and within bee individuals and taxa (Corbet et al., 1988; Buchmann and Cane, 1989; De Luca et al., 2013; Corbet and Huang, 2014; Morgan et al., 2016; Switzer and Combes, 2017; De Luca et al., 2019; Pritchard and Vallejo-Marín, 2020; Rosi-Denadai et al., 2020). If bees can increase the amplitude of their buzzes by matching the natural frequency of stamens, they will collect more pollen without necessarily expending more energy (King and Buchmann, 1996; Nunes et al., 2021). Although individual bees show variation in buzz traits, it remains unclear whether they can adjust their buzz frequency to different flower types. The sole study of natural frequency in buzz-pollinated flowers, shows that the stamen natural frequency in *Solanum laciniatum* is far below the observed frequency range of floral vibrations produced by bees (King and Buchmann, 1996), both on this species and a range of other plant species (Burkart et al., 2011; De Luca et al., 2019; Rosi-Denadai et al., 2020). Bee size may play a role in buzzing behaviour – larger bees produce buzzes with more energy (higher amplitude)(De Luca et al., 2013; Arroyo-Correa et al., 2019), whilst small bees may be unable to reach these high amplitudes and could gain more by matching their buzz frequency to the stamen natural frequency to maximise their buzz energy. A relationship between bee buzz frequency and stamen natural frequency has yet to be directly investigated.

Bees are motivated to collect as much pollen as possible, but it is in the plant's interest to act as a dispenser, distributing pollen to multiple pollinators (Harder and Thomson, 1989). Poricidal morphology contributes to this dispensing function by making it relatively difficult for an individual forager to collect all the pollen: dozens of consecutive buzzes do not release all the pollen from studied poricidal stamens (Harder and Wilson, 1994; King and Buchmann, 1996; Brito et al., 2021; Kemp and Vallejo-Marín, 2021). Buzz-pollinated plants vary widely in floral and stamen morphology (Brito et al., 2016; Melo et al., 2021), and pollen dispensing is likely shaped by biomechanical properties (King and Buchmann, 1996; Bochorny et al., 2021), yet the potential variation in stamen natural frequency has not been studied. There are two key ways to frame this variation: within-flower variation and variation between flowers of different species. Stamens can differ substantially in morphology within flowers, with distinct stamen types performing different roles (Müller, 1883; Vallejo-Marin et al., 2009). This is known as heteranthery, and it is strongly associated with the presence of poricidal anthers and the absence of nectaries (Vogel, 1978; Jesson and Barrett, 2003; Vallejo-Marin et al., 2009). There are usually two subsets of anthers: the pollinating anthers, which contribute disproportionately to pollination, and the feeding anthers, which mostly provide pollen rewards for bee visitors (Luo et al., 2008; Vallejo-Marin et al., 2009; Dellinger et al., 2019a). These types are morphologically distinct: there are fewer pollinating anthers, and they are longer and often cryptically coloured, compared to the numerous, shorter, brightly coloured feeding anthers

(Müller, 1883; Endress, 1997; Luo et al., 2009; Vallejo-Marín et al., 2010; Pinheiro-Costa et al., 2018; Dellinger et al., 2019a). Additionally, pollinating anthers are often curved to deposit pollen on specific areas, or “safe sites”, on the bee body (Bohs et al., 2007; Luo et al., 2009; Koch et al., 2017; Dellinger et al., 2019a). More subtle traits also contribute to the division of labour between the anthers, including pollen quantity (Luo et al., 2009; Pinheiro-Costa et al., 2018; Brito et al., 2021; Kemp and Vallejo-Marín, 2021), morphology (Nepi et al., 2008; Pinheiro-Costa et al., 2018), and viability (Nepi et al., 2008; Luo et al., 2009), although these differences are not universally demonstrated (Bowers, 1975; Brito et al., 2021). Importantly, pollen release rates (dispensing strategies) can also vary between long and short stamens, as shown in some species of Fabaceae and Melastomataceae (Dellinger et al., 2019a; Telles et al., 2020; Brito et al., 2021). The dispensing strategies of each type might be under different selective pressures, given their functional divergence, and many of the differences between stamen types are likely to be related to biomechanical properties, including their natural frequencies.

Stamen morphology can also differ between species, often because of differences in floral size and mating system, which are closely interrelated. Larger buzz-pollinated flowers tend to be both more attractive to bees and more reliant on them for effective pollination (Valadão-Mendes et al.; Tang and Huang, 2007; Vallejo-Marín et al., 2014). The dispensing function of poricidal stamens is presumed to be more critical for these outcrossing flowers than for flowers which can set fruit without pollinators (Vallejo-Marín et al., 2014; Kemp and Vallejo-Marín, 2021). Mating strategy may be related to pollen release rates: across six related *Solanum* taxa, smaller, selfing flowers released pollen more quickly over successive vibrations than larger, outcrossing flowers (Kemp and Vallejo-Marín, 2021). Moreover, in heterantherous lineages, the presence of selfing is associated with a reduction in heteranthery (Tang and Huang, 2007; Vallejo-Marín et al., 2014). Selfing species with small flowers are relatively weakly heterantherous, with less disparity in stamen size, morphology, and colour (Tang and Huang, 2007; Vallejo-Marín et al., 2014). If bee species adjust their buzzes to different flower types, then potential matching of the stamen natural frequency could differentially affect pollen release, depending on the flower size or mating system of the species.

In this study, I measure the natural frequency of stamens of six *Solanum* taxa, which vary in size, morphology, and mating strategy: three taxa are large-flowered, highly heterantherous and outcrossing, and three are small-flowered, weakly heterantherous and selfing. These natural frequencies have been measured using laser vibrometry by Nunes *et al.* (2021), but here I use an alternative method employed by King and Buchmann (1995) and Timerman *et al* (2014). One advantage of laser vibrometry is the potential for high precision in measurements; however, it requires specialist lab equipment. The method I use here is relatively cheap and could be performed under field conditions. This method explicitly applies cantilever theory to stamens: a stamen is comparable to a cantilever beam, an object fixed at its base, with the other end free to move (Niklas, 1992; King and Buchmann, 1995; Timerman et al., 2014). I calculate natural frequency from a combination of stamen morphological traits and measurements of the displacement of anthers under varying weights. I ask the following questions: 1. How does the natural frequency of stamens vary between contrasting anther types? 2. How does the natural frequency of each type of stamen vary between different mating systems? 3. How do these measurements of natural frequency compare to those reported by Nunes et al.(2021)?

3.3 Methods

3.3.1 Plant material

I studied six taxa of *Solanum* section *Androceras*, which comprise three pairs of sister taxa*:* *S. rostratum* Dunal and *S. fructo-tecto* Cav.; *S. citrullifolium* A.Braun and *S. heterodoxum* Dunal; and *S. grayi* Whalen var. *grandiflorum* Whalen and *S. grayi* var. *grayi* Whalen (Stern et al., 2010) (Figure 3.1). All the taxa are native to the southern US and northern Mexico but differ in their distribution (Whalen, 1978; Stern et al., 2010; Vallejo-Marín et al., 2014). *Solanum* species are buzz-pollinated and have poricidal anthers, requiring bee vibrations for effective pollination, and are nectarless – pollen is the only reward for bee visitors (Bowers, 1975; Whalen, 1979). All taxa are self-compatible (Vallejo-Marín et al., 2014), but three of them are large-flowered, highly heterantherous and predominantly outcrossing, whilst three are small-flowered, weakly heterantherous and set fruit without pollinators in the glasshouse (pers. obs.). *S. rostratum* is the only species with published pollinator observations and is visited regularly by buzzing bees of different sizes, including in the genera *Bombus, Centris, Lasioglossum*, and *Xylocopa* (Bowers, 1975; Solís-Montero et al., 2015). It is likely that the other large-flowered taxa share these pollinators, but little is known about the visitors to the small-flowered taxa.

Plants were grown in the University of Stirling glasshouses in the spring of 2019. Seeds of *S. citrullifolium* were obtained from the Radboud Botanic Gardens and all other seeds were collected from wild populations in Mexico. Seeds were treated for 24hr with a 1000ppm solution of gibberellic acid (GA3: Sigma-Aldrich, Dorset, UK) to induce germination, following Vallejo-Marin et al. (2014). Seeds were planted in modular seed compost (William Sinclair Horticulture PLC, Lincoln, UK) in seed trays, and kept in glasshouses. Glasshouses were supplemented with artificial fluorescent lighting (16 hours of daylight), and supplemental heating was provided if temperature dropped below 16°C. Seedlings were transplanted to 1.5L pots in a mix of All Purpose Growing Medium and Perlite (4:1 ratio, William Sinclair Horticulture PLC, Lincoln, UK). Plants were kept in a pollinator-proof glasshouse, and fertilised weekly with Tomorite (Levington, Surrey, UK)

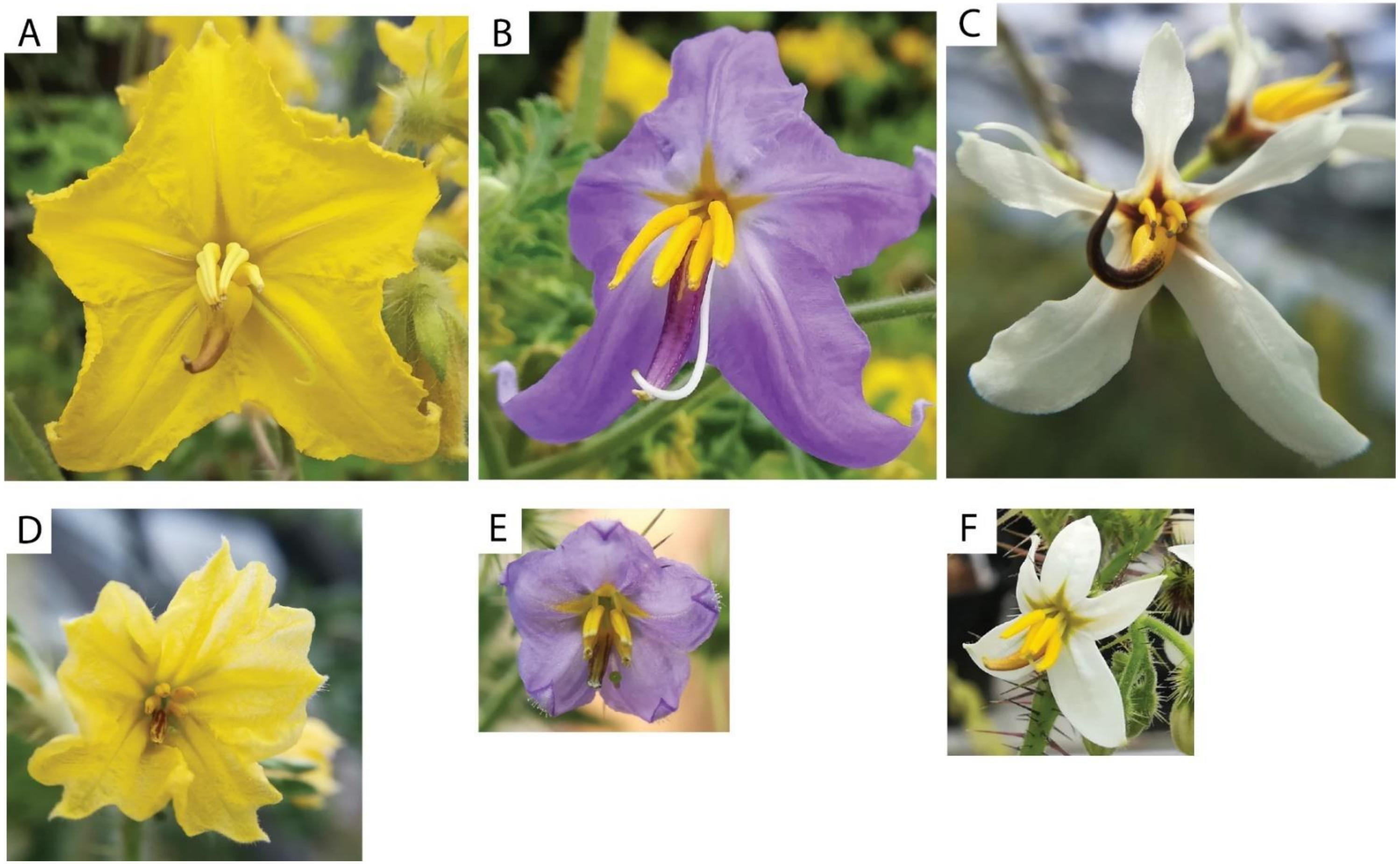

Figure 3.1 Six *Solanum* taxa used in measurements of natural frequency. Three taxa (A-C) are large, outcrossing, and highly heterantherous: *S. rostratum*, *S. citrullifolium*, *S. grayi grandiflorum*. Three (D-F) are small-flowered, selfing, and weakly heterantherous: *S. fructo-tecto, S. heterodoxum*, *S. grayi grayi*. Photos provided by D. Moore.

3.3.2 Measurement of floral traits

Flowers were collected from the glasshouses in the morning of experiments and kept in floral foam. Between 17-21 flowers of each taxon were used. Prior to displacement experiments, individual whole flowers (without stem) were weighed. After experiments (see below), stamens used for the experiment were weighed and measured: one pollinating and one feeding stamen for each flower. In the case of *S. heterodoxum*, multiple feeding stamens were weighed together as they are extremely light. I used callipers to measure stamen lengths.

3.3.3 Measurement of flexural rigidity and first natural frequency

Using the same flowers as above, for each flower, I removed the corolla and attached the receptable to a clamp using forceps. I photographed the pollinating stamen against a background of graph paper for later digital measurements (the distance of flower from the paper was kept constant across the whole experiment) (Figure 3.2). A coil of copper wire of known mass was hung from the pollinating stamen, as close as possible to a third of the length from the anther tip, and this was again photographed (Figure 3.2). To aid visibility, I then removed the pollinating stamen and three feeding stamens and repeated the procedure for one feeding stamen. I analysed the photos in ImageJ (Schneider et al., 2012) to extract displacement values (y), by comparing the position of the anther on the grid (graph paper) without the coil to its position with the coil. This method is adapted from King and Buchmann (1995) and Timerman *et al.* (2014).

I used the following variables in the calculation of flexural rigidity and first natural frequency (all lengths, distances and masses converted to SI base units, i.e. m or kg from mm and g) (Figure 3.2):

W:	Weight of the copper wires added to anther (kg).
y:	Displacement at the point of the weight attached to the anther (m).
L:	Distance from the added weight to the attached end of the filament (m).
m:	Mass of the stamen (kg).

To calculate flexural rigidity (EI, N·m²), I used the following equation, $\mathrm{EI} = \frac{W 9.8 L^3}{3y}$ ((Timerman et al., 2014), Timerman correspondence). The flexural rigidity of an object is based on the cantilever model and reflects its resistance to bending, with higher values representing higher resistance (Niklas, 1992).

To calculate first natural frequency (Fn, Hz), I used Fn = $\frac{0.5\pi\sqrt{3EI}}{mL^3}$ ((Timerman et al., 2014), Timerman correspondence). Broadly, this equation takes stiffness (flexural rigidity) as the numerator and mass as the denominator. An increase in flexural rigidity therefore increases natural frequency, while an increase in mass decreases the natural frequency (Niklas, 1992).

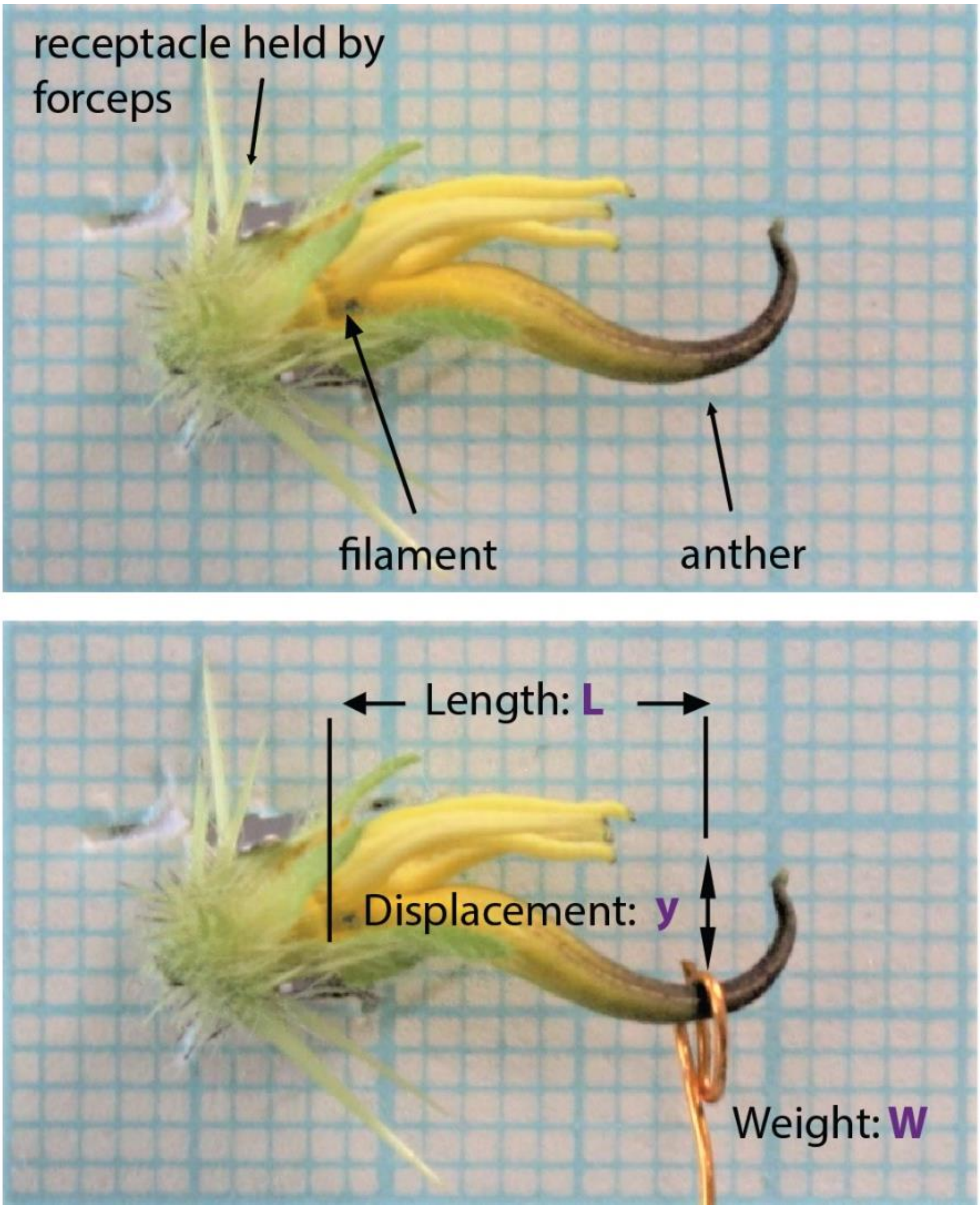

Figure 3.2 Set-up of experiment to measure the displacement of stamen by a known weight, for the calculation of flexural rigidity and first natural frequency. The pollinating anther of S. citrullifolium is shown here.

3.3.4 Statistical analysis

To assess the relationship between flower type (large or small, also a proxy for mating system), stamen type (pollinating or feeding) and stamen length, I fitted a linear mixed model with stamen length (mm) as the response variable, an interaction term between flower type (small and selfing vs large and outcrossing) and stamen type, and plant species as a random effect. Stamen length was transformed (square-rooted) to account for heterogeneity of variance. Statistical significance was assessed with F values calculated using Type III ANOVA, comparing the model with the null hypothesis, and Satterthwaite's correction, to account for differences in sample variances.

To analyse variation in flexural rigidity, I fitted a linear mixed model with rigidity as the response, stamen length (m), flower type, and anther type as fixed effects, and plant species as a random effect. Statistical significance was assessed with F values calculated using Type II ANOVA and Satterthwaite's correction.

To analyse variation in natural frequency, I fitted a linear mixed model with natural frequency as the response, stamen rigidity, length (m), flower type, and anther type as fixed effects, and plant species as a random effect. Statistical significance was assessed with F values calculated using Type II ANOVA and Satterthwaite's correction.

Linear models were fitted using the package *lme4* (Bates et al., 2015). Model diagnostics were performed using the package *DHARMa* (Hartig, 2019). All statistical analysis was performed in R 4.0.2 (R Development Core Team, 2021).

3.4 Results

3.4.1 Morphological traits

Whole-flower mass (g) ranged from 0.018 (*S. heterodoxum*) to 0.165 (*S. rostratum*) (Table 3.2, Figure 3.3B). Stamen mass (g) ranged from 0.0003 (feeding stamen of *S. fructo-tecto*) to 0.029 (pollinating stamen of *S. rostratum*) (Table 3.2, Figure 3.3B). Stamen length (mm) varied from 3.23 (feeding stamen of *S. heterodoxum*) to 16.98 (pollinating stamen of *S. rostratum*) (Table 3.1A, Figure 3.3C). I found a significant interaction effect between mating system and stamen type on stamen length: small, selfing flowers have less disparity in stamen lengths than large, outcrossing flowers (estimate: 0.47±0.05, $p<0.001$, $n=120$) (Figure 3.3D, Table 3.1A). I also found separate effects of stamen type: feeding stamens are shorter than pollinating stamens (estimate: -0.63±0.03, $p<0.001$, $n=120$), and mating system: selfing flowers have shorter stamens than outcrossing flowers (estimate: -1.33±0.22, $p<0.01$, $n=120$) (Table 3.1A).

3.4.2 Stamen flexural rigidity

Stamen length had a positive effect on flexural rigidity (stiffness), with longer anthers being stiffer (estimate: 433.3±49.67, $p<0.001$, $n=120$) (Table 3.1B, Figure 3.4). Stamen type also has an additional effect on stiffness: feeding stamens are less stiff than pollinating stamens across all species (estimate: -1.17±0.17, $p<0.001$, $n=120$) (Table 3.1B, Figure 3.4). There is no effect of mating system (Table 3.1B, Figure 3.4).

3.4.3. Stamen natural frequency

Stamen natural frequencies were lowest in the pollinating stamens of *S. citrullifolium*, 86.8±5.2Hz, and highest in the feeding stamens of *S. heterodoxum*, 203±16.6Hz (Figure 3.5). I found a significant negative effect of stamen length on natural frequency: longer stamens have lower natural frequencies than shorter stamens (estimate: -8.32±2.78, $p<0.01$, $n=120$) (Table 3.1C, Figure 3.5). There is also a significant positive effect of flexural rigidity: stiffer stamens have higher natural frequencies (estimate: 8.36±2.29, $p<0.0001$, $n=120$) (Table

3.1C, Figure 3.5). When these effects are accounted for, there is no effect of stamen type or mating system (Table 3.1C, Figure 3.5).

	Estimate	**Std. error**	***P*-value**
A. Stamen length			
Stamen type (feeding)	-0.63	0.03	**<0.001**
Mating system (selfing)	-1.33	0.22	**<0.01**
Stamen:mating (feeding:selfing)	0.47	0.05	**<0.001**
B. Flexural rigidity			
Length	433.3	49.67	**<0.001**
Stamen type (feeding)	-1.17	0.17	**<0.001**
Mating system (selfing)	-0.83	0.57	0.19
C. Natural frequency			
Length	-8.32	2.78	**<0.01**
Flexural rigidity	8.36	2.29	**<0.001**
Stamen type (feeding)	-4.2	8.14	0.61
Mating system (selfing)	2.2	1.88	0.27

Table 3.1 Parameter estimates of the three separate linear models fitted with either stamen length, flexural rigidity or natural frequency as a response, and stamen type, mating system, stamen length and/or flexural rigidity as explanatory variables. P-value in model A (with interaction term) calculated using Type III sums of squares. P-value in models B and C calculated using Type III sums of squares. Sample size is 120 for all models.

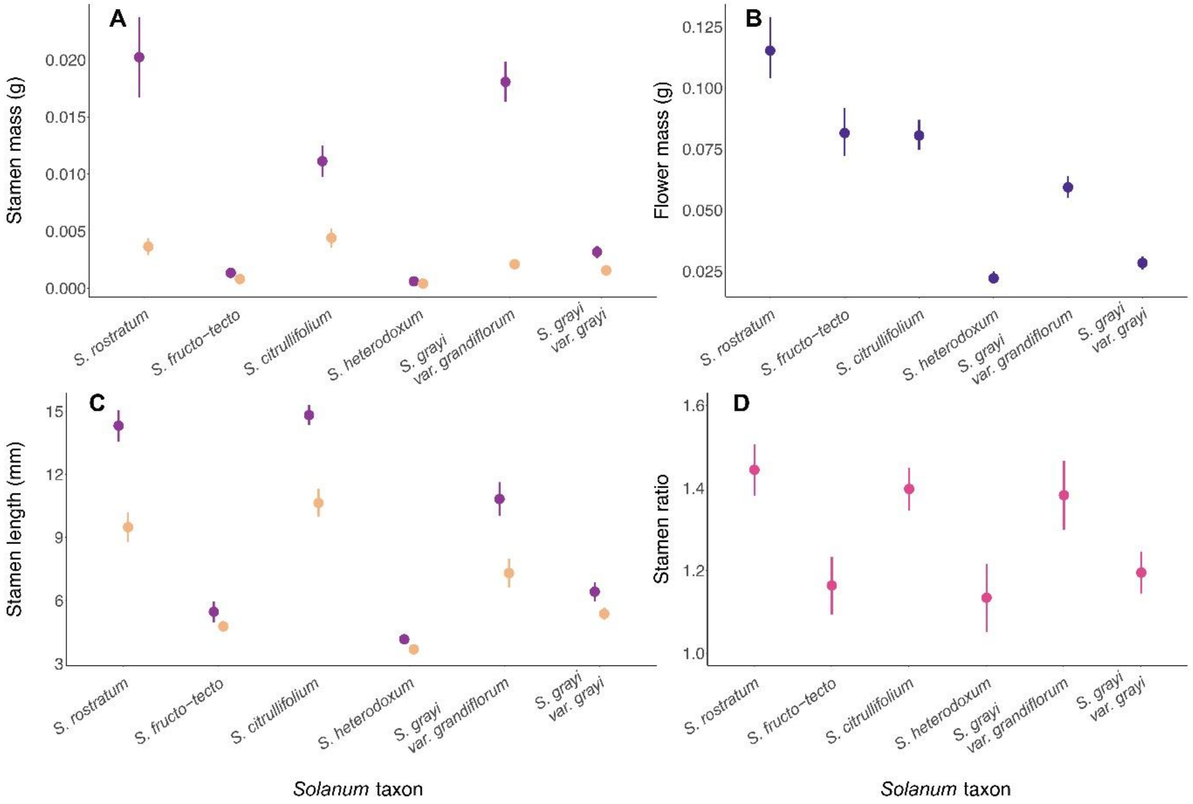

Figure 3.3 Floral morphological traits for six *Solanum* taxa. Pollinating stamens are shown in purple; feeding stamens are shown in yellow. Large-flowered species are indicated with circles; small-flowered species are indicated with diamonds. A: stamen mass (g); B: whole flower mass (g); C: stamen length (mm); D: stamen ratio – pollinating stamen length/feeding stamen length. See Table 3.4.1 for sample sizes.

	Solanum rostratum	***S. fructo-tecto***	***S. citrullifolium***	**S. *heterodoxum***	***S. grayi var. grandiflorum***	**S. *grayi var. grayi***
Flower mass (g)	0.118 ± 0.003	0.093 ± 0.006	0.086 ± 0.002	0.020 ± 0.001	0.06 ± 0.002	0.028 ± 0.001
Pollinating stamen (PS) mass (g)	1.78E-02 ± 1.14E-03	1.42E-03 ± 6.86E-05	1.05E-02 ± 4.70E-04	4.66E-04 ± 3.52E-05	1.61E-02 ± 8.22E-04	2.89E-03 ± 1.65E-04
Feeding stamen (FS) mass (g)	3.45E-03 ± 1.81E-04	8.95E-04 ± 5.92E-05	4.24E-03 ± 2.30E-04	3.66E-04 ± 1.87E-05	2.03E-03 ± 7.72E-05	1.43E-03 ± 1.03E-04
PS length (mm)	14.301 ± 0.177	6.150 ± 0.194	15.1065 ± 0.182	4.259 ± 0.108	10.640 ± 0.199	6.271 ± 0.145
FS length (mm)	9.584 ± 0.185	5.716 ± 0.232	11.231 ± 0.233	3.661 ± 0.102	7.486 ± 0.194	5.403 ± 0.084
n	21	21	20	20	21	17

Table 3.2 Floral morphological measurements for six *Solanum* taxa (mean and standard error): flower mass, stamen mass and stamen length for each stamen type. N = number of flowers measured.

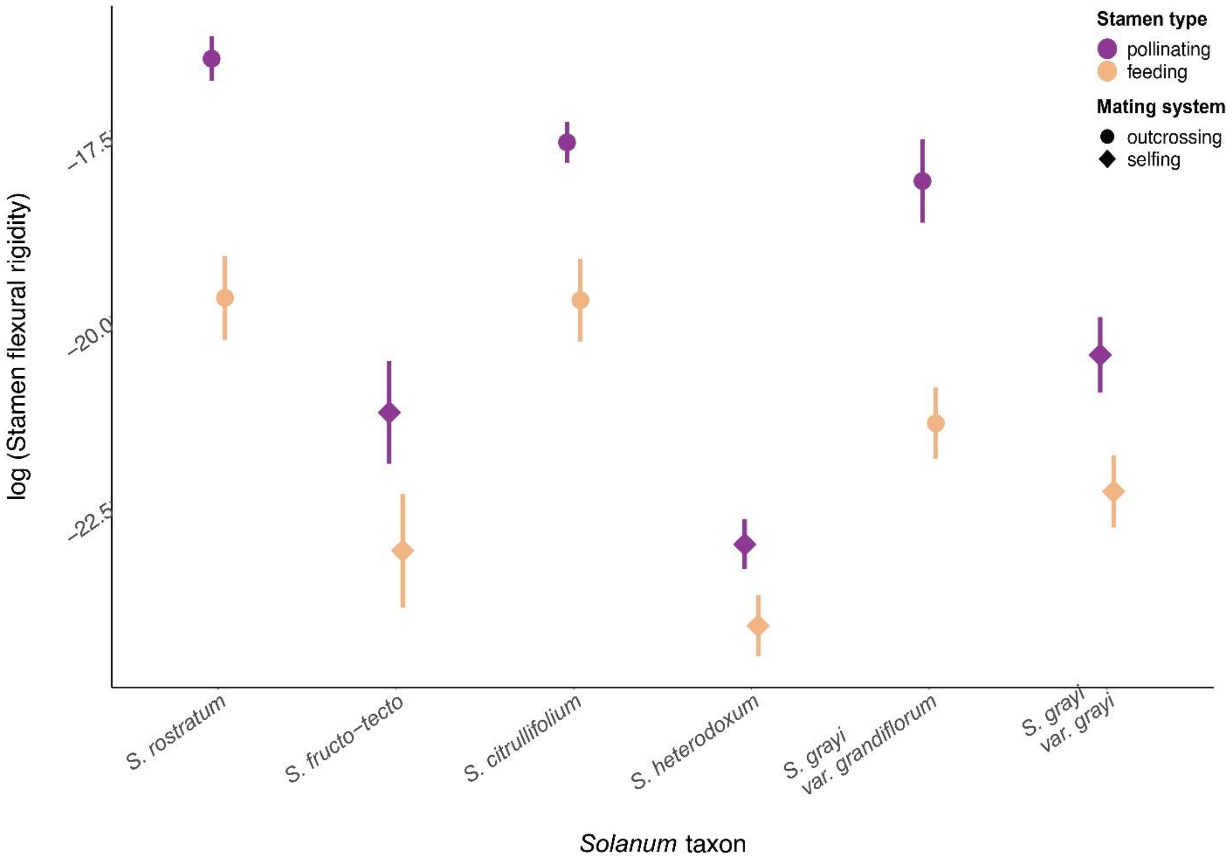

Figure 3.4 Stamen flexural rigidity (log-transformed) in six Solanum taxa. Pollinating stamens are shown in purple; feeding stamens are shown in yellow. Large-flowered species are indicated with circles; small-flowered species are indicated with diamonds.

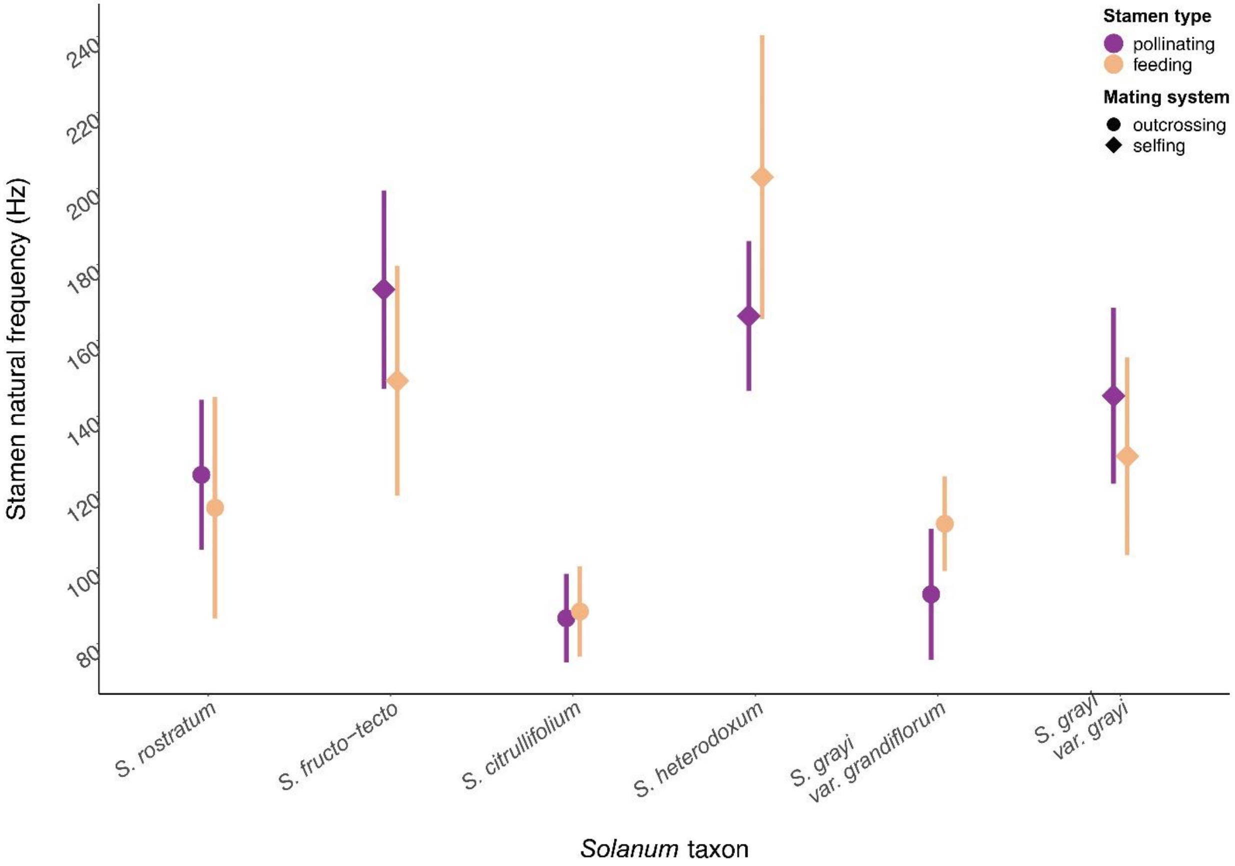

Figure 3.5 Stamen first natural frequency (Hz) in six Solanum taxa. Pollinating stamens are shown in purple; feeding stamens are shown in yellow. Large-flowered species are indicated with circles; small-flowered species are indicated with diamonds.

3.5 Discussion

Stamens of buzz-pollinated plants are expected to vary in their natural frequency, due to morphological trait differences, and this variation may have implications for both pollen release and bee foraging behaviour. Most studies of natural frequency have been conducted in wind-pollinated plants (Timerman et al., 2014; Timerman and Barrett, 2018, 2019). Here, in six buzz-pollinated and heterantherous *Solanum* taxa, I found that stamen natural frequency varies with mating system: outcrossing taxa have lower natural frequencies, as found by Nunes *et al*.(2021). This effect can be explained by differences in stamen length and stiffness. I found no consistent effect of stamen type on natural frequency, although stamen stiffness was affected by stamen type: even when accounting for length, pollinating stamens are stiffer than feeding stamens.

I calculated stamen natural frequencies to range from around 90Hz to 200Hz, compared to the overlapping but wider range found by Nunes et al. (2021): 65 to 300Hz. Similarly, in the only other natural frequency study in *Solanum*, King and Buchmann (1996) found *S. laciniatum* stamens to have a natural frequency of 143Hz, in the middle of the range found here. Prior to Nunes et al. (2021) and this study, no one has investigated the variation of natural frequency in closely related species with different morphologies and mating systems. Here, the stamen natural frequencies of large-flowered outcrossing species are consistently lower than those of small-flowered species: *S. heterodoxum* stamens are more than double the natural frequency of the stamens of the closely related *S. citrullifolium*. Nunes *et al*. (2021) found the same trend, although the disparity is not so extreme, and individual taxa differ in their natural frequencies between studies. I found that the difference between taxa is explained by length: length is inversely related to natural frequency and larger flowers have longer stamens. Despite differences in stiffness (larger stamens are also stiffer), length drives these low natural frequencies. Nunes *et al.* (2021) found that morphological traits, including length, could not fully explain the variation in natural frequency, suggesting that unknown properties also play a role – for instance, they did not measure stiffness.

Previous studies have not compared stamen types within a heterantherous flower. Nunes *et al*. (2021) found that natural frequency varied with stamen type: feeding stamens have higher natural frequencies than pollinating stamens. I did not find this effect – stamens were relatively similar in most of the flower types. I did find that feeding stamens were consistently less stiff than pollinating stamens, likely due to pollinating stamens being thicker. One explanation for the discrepancy between the two studies is the stamen shape – in the method I used, the cantilever model assumes the stamen to be a straight beam (King and Buchmann, 1995; Timerman et al., 2014). However, the pollinating anthers of many of the species are curved in an S-shape which may well affect the accuracy of their calculated frequencies (Vallejo-Marín et al., 2014). In previous uses of this method, such as in *Rhododendron augustinii* (King and Buchmann, 1995) and the wind-pollinated *Plantago lanceolata* (Timerman et al., 2014), stamens are relatively straight. Furthermore, the filament in those species is longer, with a short anther (concentrated mass) at the end (King and Buchmann, 1995; Timerman et al., 2014), whereas the *Solanum* stamens studied here are mainly anther, with an unevenly distributed mass particularly in the pollinating stamens, and a very short filament. These differences are likely to affect the point at which the stamen bends, affecting the measurement of the natural frequency. Advances in stamen modelling will enable us to account for variation in stamen morphologies and estimate biomechanical properties with increased accuracy and precision (Jankauski et al., 2021, preprint).

Biomechanical properties affect the function of the stamen as a pollen dispenser, and the difference in natural frequency between large, outcrossing flowers and small, selfing flowers may contribute to their respective mating strategies. The lower natural frequencies in larger flowers, some lower than 100Hz, may be too low for bees to match, preventing individuals from releasing too much pollen and ensuring some is left for future visitors (Harder and Thomson, 1989; King and Buchmann, 1996). The higher natural frequencies in small species are closer to bee vibrations, which may facilitate pollen release, and thus reproduction, even on a single visit. Although I did not find a difference between pollinating and feeding stamens here, the difference found by Nunes et al. (2021) may contribute to the division of labour in heterantherous flowers. Pollinating stamens have lower natural frequencies, further outside the range of bee vibrations, while bees may more easily exploit the higher natural frequencies in the feeding stamens (Nunes et al., 2021).

The significance of stamen natural frequency for buzz pollinated plants depends largely on its relationship with bee buzzing behaviour. The range of stamen natural frequencies found here overlaps with the low end of the range of bee floral vibrations: 100-400Hz (Burkart et al., 2011; De Luca et al., 2019; Rosi-Denadai et al., 2020). Theoretically, bees could exploit resonance by adjusting their buzzes to the natural frequency of the flower they are foraging from. There is little evidence that individual bees change their floral vibration frequency with species, but Switzer and Combes (2017) found that *Bombus impatiens* individuals buzzed at different frequencies on different *Solanum* spp. Interestingly, they buzzed at higher frequencies on the large-flowered species in their study, which is likely to have a lower stamen natural frequency, based on the relationship between natural frequency and stamen length. This suggests that the difference in bee behaviour is not accounted for by resonance. Most studies on individual buzzing behaviour have focused on medium-sized or large bee species, such as *Bombus* (Switzer and Combes, 2017; Arroyo-Correa et al., 2019) and *Xylocopa* (Burkart et al., 2014), but smaller bees may benefit most from exploiting stamen resonance, if they are otherwise unable to reach high buzz amplitudes. This may be particularly effective on large, heterantherous flowers, on which small bees often grasp only one or two anthers while buzzing (Solís-Montero et al., 2015).

An important caveat to the present study is that I calculated the natural frequency of freely moving stamens in isolation without the interference of a bee, which would be likely to affect natural frequency. To my knowledge, only two studies have investigated this possibility: King and Buchmann (1996) found that adding a bee-equivalent mass to their *S. laciniatum* stamens did not significantly affect natural frequency, but a recent study using finite element modelling, based on *S. elaeagnifolium* stamens, suggest a negative effect of bee mass on natural frequency, with large bees reducing the natural frequency further than smaller bees (Jankauski et al., 2021, preprint). This effect will be complicated by the position of the bee on the flower, which can range from buzzing one stamen to grasping all stamens together, and variation in this behaviour is itself related to the size match between the bee and flower, as discussed above (Luo et al., 2009; Solís-Montero et al., 2015). Furthermore, in heterantherous flowers, bees commonly grasp the group of feeding anthers, leaving the pollinating anther relatively free to vibrate (Luo et al., 2009; Vallejo-Marin et al., 2009). The relevance of bee size to stamen natural frequency requires detailed investigations on multiple taxa with different stamen morphologies and arrangements, integrated with observations of bee foraging behaviour.

The cantilever model used here simplifies stamen characteristics relative to empirical measurements using laser vibrometry (Nunes et al., 2021). Yet despite not fully capturing the variation in stamen morphology within heterantherous flowers, my results overall provide similar frequency values to those found by Nunes et al. (2021), showing that stamen natural frequency varies between buzz-pollinated species with differing floral morphologies and mating systems. The relevance of this variation to either bee foraging behaviour or plant reproduction remains an open question.

Chapter Four: Bumblebee buzzing behaviour in two morphologically distinct *Solanum* species

Part of this chapter (buzz frequencies of ten focal bees) has been published in:

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4.1 Abstract

The production of floral vibrations in pollen foraging is energetically costly for bees, who are motivated to reduce these costs while maximising pollen collection. A potential strategy is to exploit the biomechanical properties of flowers by matching buzz frequency to stamen natural frequency. Vibrating a stamen at its natural frequency amplifies the vibrations through resonance, potentially increasing pollen release. Stamen natural frequency differs between buzz-pollinated species, and we might expect bees to adjust their buzz characteristics accordingly. Here, I investigate the effects of plant species and handling experience on the peak frequency and duration of *Bombus terrestris* floral vibrations. I compare buzzes from multiple visits by individually marked bees on two plant species which differ in stamen natural frequency and pollen release rates: the large-flowered, *Solanum citrullifolium,* and the small-flowered *S. heterodoxum*. I find that bees do not tune buzz frequency to stamen natural frequency, but buzz *S. heterodoxum* at slightly higher frequencies, and invest less time in buzzing these flowers. I suggest that bees are making small alterations to their behaviour in response to differences in availability and pollen release rates between plant species.

4.2 Introduction

Our understanding of bee cognition and foraging behaviour has advanced rapidly in the last few decades (Perry et al., 2017), yet many studies of foraging behaviour have focused on nectar foraging abilities (Nicholls and Hempel de Ibarra, 2017). Pollen foraging is relatively understudied until recently, and the active collection of pollen typically requires more skill and behavioural flexibility than collecting nectar (Raine and Chittka, 2007; Nicholls and Hempel de Ibarra, 2017). One of the main pollen foraging techniques used by nearly half of all bee species is the production of floral vibrations (Cardinal et al., 2018; Vallejo-Marín, 2021), which are required for the collection of pollen from poricidal anthers (Michener, 1962; Buchmann, 1983). Floral vibrations are efficient compared to other collection methods, yet they are also energetically costly to produce (Russell et al., 2017). Bees should therefore benefit from adjusting their buzzing behaviour to release as much pollen as possible whilst minimising energy expenditure.

Bees are known to learn and remember associations between pollen sources and floral traits, such as colour (Nicholls and de Ibarra, 2014; Muth et al., 2016) and scent (Konzmann and Lunau, 2014; Nicholls and Hempel de Ibarra, 2017; Nery et al., 2020). They can also detect a range of more subtle properties, including texture (Kevan and Lane, 1985), electric fields (Clarke et al., 2013), temperature (Harrap et al., 2017), and humidity (Harrap et al., 2021), although the relationship of these properties with pollen foraging is unknown. The ability of bees to perceive biomechanical properties and/or associate them with pollen rewards has not been investigated, but bees do respond to pollen release (Russell et al., 2016b; Switzer et al., 2019). Bees may be able to increase pollen release by matching their floral vibrations to the traits of buzz-pollinated flowers. Specifically, because of the tight relationship between vibrations and pollen release, bees may be able to exploit floral vibrational properties to increase pollen release (King and Buchmann, 1996; Rosi-Denadai et al., 2020; Nunes et al., 2021). One of these properties is the stamen natural frequency, the frequency at which the stamen oscillates when disturbed by external forces: vibrations are amplified when they match this natural frequency (Niklas, 1992; Timerman et al., 2014). Given that the amplitude of floral vibrations is positively related to pollen release (Harder and Barclay, 1994; De Luca et al., 2013; Rosi-Denadai et al., 2020; Kemp and Vallejo-Marín, 2021), frequency-matching may provide a way for bees to increase foraging efficiency without incurring extra energetic costs. Buzz-pollinated species differ in their stamen natural frequencies (King and Buchmann, 1996; Nunes et al., 2021), yet it is not known whether bees adjust their vibrations to exploit these differences.

Bees may alter their buzz characteristics in response to pollen release, perhaps prompting a change in buzz frequency between flower types. Bumblebees (*B. impatiens*) have been found to increase their buzz frequency when vibrating flowers which do not release pollen (Russell et al., 2016b; Switzer et al., 2019). It is therefore possible that bees respond to different rates of pollen release across plant species. Buzz-pollinated plants differ in their dispensing strategies, i.e. the rate at which pollen is released over time (Dellinger et al., 2019a; Kemp and Vallejo-Marín, 2021). A slow dispensing rate is particularly beneficial for outcrossing plants, which benefit most from their pollen being carried by multiple pollinators (Harder and Wilson, 1994). Selfing plants may not require bee visitors, or one bee visit may be enough to set fruit successfully in plants with facilitated selfing strategies (Tang and Huang, 2007; Vallejo-Marín et al., 2014). As expected, pollen dispensing curves differ between outcrossing and selfing buzz-pollinated plants: in a comparison of six *Solanum* taxa, Kemp and Vallejo-Marin (2020) found that large-flowered, outcrossing species release pollen more slowly (a shallower curve) than small-flowered, selfing species at the same frequency and amplitude of artificial vibration (Vallejo-Marín et al., 2014; Kemp and Vallejo-Marín, 2021). In these taxa, selfing species also contain far fewer pollen grains in total (Kemp and Vallejo-Marín, 2021). As a higher proportion of this small amount is released in the first few buzzes, this could impact on pollen release during the latter stages of a bee’s visit. It is possible that bees respond to this drop-off in pollen release by adjusting their buzz characteristics, for instance increasing their buzz frequencies (Switzer et al., 2019).

Evidence that bees adjust their buzz frequency to different floral morphologies is limited. One field study found that the carpenter bee *Xylocopa frontalis* produces buzzes of different frequency on different species of *Solanum*, but they did not find the same difference for *X. suspecta* (Burkart et al., 2011). Corbet and Huang (2014) similarly found differences in *B. friseanus* buzz frequency and duration between several *Pedicularis* species and hypothesised that these were linked to morphological traits, such as the galea shape (Corbet and Huang, 2014). However, neither of these studies could ascertain that the same subset of individuals was visiting each species: in the *Pedicularis* study, bees tended to assort by size amongst flowers, confounding any potential effect of flower type on buzz properties (Corbet and Huang, 2014). Few studies have evaluated the vibrations of individually marked bees on different species. Meanwhile, Switzer and Combes (2017) provide the clearest evidence for an effect of plant species on buzz frequency: *Bombus impatiens* individuals buzz at higher frequencies on *S. carolinense* than on *S. lycopersicum* or *S. dulcamara* and at longer durations on *S. dulcamara* than the other species. Interestingly, *S. carolinense* produces significantly larger flowers than the other two species, ~3cm vs. ~1cm in corolla diameter, and has a different stamen architecture: unfused anthers rather than a tight cone (Switzer and Combes, 2017). Both flower size and the fusion of anthers may the transmission of vibrations across the flower and the release of pollen (Nevard et al., 2021; Vallejo-Marín et al., 2021). On the other hand, Switzer et al. (2019) found that bees do not change buzz frequency on *S. dulcamara* flowers with experimentally altered masses. Changes in floral mass are likely to affect the natural frequency of the flower (Jankauski et al., 2021, preprint).

Here, I assess the effect of plant species and handling experience on bumblebee (*Bombus terrestris*) buzz frequency and duration in two morphologically different species of buzz-pollinated *Solanum*. I selected the closely related species *Solanum citrullifolium* and *S. heterodoxum* for their marked differences in size, morphology, and mating strategies (Vallejo-Marín et al., 2014). *S. citrullifolium* is large, highly heterantherous and dependent on pollinator-mediated outcrossing for effective pollination (Vallejo-Marín et al., 2014). *S. heterodoxum* is small, weakly heterantherous and readily sets fruit without pollinators (Vallejo-Marín et al., 2014). The two species also differ substantially in both total pollen volume and pollen release rates: *S. citrullifolium* contains 10 times as many pollen grains as *S. heterodoxum* (Kemp and Vallejo-Marín, 2021). After 30 vibrations, more than 80% of this pollen remains in *S. citrullifolium*, while less than 40% of the pollen in *S. heterodoxum* remains (Kemp and Vallejo-Marín, 2021). The two flower types differ in stamen natural frequency: the stamens of *S. citrullifolium* have a natural frequency of around 45-65Hz, while those of *S. heterodoxum* are around 120Hz (Chapter Three) (Nunes et al., 2021).

I ask the following questions: 1. How does bumblebee body size affect buzz characteristics? 2. Do bumblebee buzzes change in frequency or duration between plant species? If bees are exploiting stamen natural frequency, I expect that they will perform lower-frequency vibrations on *S. citrullifolium*. 3. Do buzzes change within single visits to either species? If bees change their buzzes gradually during a single visit, they may be responding to feedback from the flower, for instance pollen release. 4. Do buzzes change with experience across multiple visits to either species?

4.3 Methods

4.3.1 Plant material

I performed experiments using two closely related species of *Solanum* section *Androceras: S. citrullifolium* A.Braun and *S. heterodoxum* Dunal (Whalen, 1979; Stern et al., 2010). *Solanum* species are buzz-pollinated and have poricidal anthers, requiring bee vibrations for effective pollination, and are nectarless: pollen is the only reward for bee visitors (Bowers, 1975). The two species share a native range from the southern US to northern Mexico but differ in their distribution within the range and in floral morphology and reproductive strategy (Whalen, 1978; Stern et al., 2010; Vallejo-Marín et al., 2014).

S. citrullifolium produces relatively large purple flowers, which are highly heterantherous - they possess two anther types (Bohs et al., 2007; Vallejo-Marín et al., 2014). The average corolla width of these flowers is approximately 26mm (Nunes et al., 2021). The single pollinating anther contributes disproportionately to pollination and is large and S-shaped with somewhat cryptic colouration (pale purple or pale yellow). The four feeding anthers contribute more to providing pollen for visiting bees, and are shorter, straight, and highly conspicuous (bright yellow). Flowers of this species are also enantiostylous: the style is deflected to either left or right in each flower, with the pollinating anther on the opposite side (Jesson and Barrett, 2002; Vallejo-Marín et al., 2014). *S. citrullifolium* is self-compatible but presumed to be predominantly outcrossing, based on its floral morphology and relatively high pollen:ovule ratios (Vallejo-Marín et al., 2014). To our knowledge, no pollinator observations have been published for this species, but large-flowered relatives in *Solanum* sect *Androceras* are visited by a variety of buzz-pollinating bees, including the genera *Bombus, Centris, Lasioglossum and Xylocopa* (Bowers, 1975; Solís-Montero et al., 2015). A bee visitor will typically buzz the horizontal flower in an upright position, grasping the feeding anthers and positioned such that the pollinating anther is curved towards her dorsal side (Vallejo-Marin et al., 2009).

S. heterodoxum produces considerably smaller purple flowers, with shorter anthers and less differentiation between anther types than in *S. citrullifolium*. The average corolla width of these flowers is approximately 11mm (Nunes et al., 2021). It is self-compatible, has a relatively low pollen:ovule ratio, and readily sets fruit in glasshouses without pollinators (personal observations) (Vallejo-Marín et al., 2014). Little is known of its pollination ecology in the wild, but bumblebees (*B. terrestris*) in the laboratory are capable of buzzing the flowers to collect pollen (personal observations).

Plants were grown in the University of Stirling glasshouses in the spring of 2018. Seeds of *S. citrullifolium* were obtained from the Radboud Botanic Gardens and seeds of *S. heterodoxum* were collected from wild populations in Mexico. Following Vallejo-Marin et al. (2014), seeds were treated for 24h with a 1000ppm solution of gibberellic acid (GA3: Sigma-Aldrich, Dorset, UK) to induce germination. They were planted in seed trays using modular seed compost (William Sinclair Horticulture PLC, Lincoln, UK), and kept in the university glasshouses. Glasshouses were supplemented with artificial fluorescent lighting (16 hours of daylight), and supplemental heating was provided if temperatures dropped below 16°C. Seedlings were transplanted to 1.5L pots in a mix of All Purpose Growing Medium and Perlite (4:1 ratio, William Sinclair Horticulture PLC, Lincoln, UK). Plants were kept in a pollinator-proof glasshouse, and fertilised weekly with Tomorite (Levington, Surrey, UK).

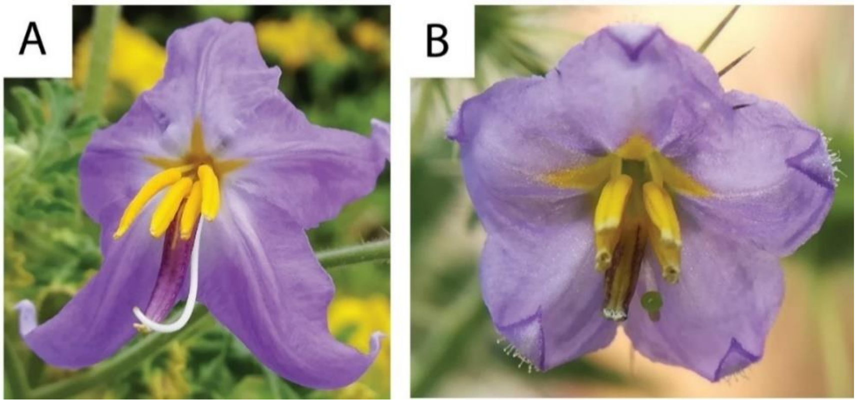


Figure 4.1 Two *Solanum* species used in experiments on bumblebee buzzing behaviour. *Solanum citrullifolium* (A) is large-flowered, outcrossing and highly heterantherous with an average corolla width of 26mm. *Solanum heterodoxum* is small-flowered, selfing and weakly heterantherous, with an average corolla width of 11mm.

4.3.2 Bees

I performed behavioural experiments using workers from two commercially obtained buff-tailed bumblebee colonies (*Bombus terrestris audax*) (Biobest, supplied by Agralan Ltd, Swindon, UK). Colonies were provisioned with Biogluc sucrose solution underneath the colony *ad libitum* (Biobest Group NV, Westerlo, Belgium) and ground honeybee-collected pollen (Agralan Ltd, Swindon, UK). Room temperature was 22-23°C and relative humidity was 50-60%. *B. terrestris* is not a natural pollinator of our two *Solanum* species, but is comparable in size and behaviour to the natural pollinators of *Solanum* section *Androceras*, which include *Bombus* spp (Solís-Montero et al., 2015). *Bombus terrestris* is also a widely used model organism for the study of bee behaviour and pollination ecology.

4.3.3 Experimental set-up

Bumblebee colonies were attached to a grey-painted flight arena (60×60×37 cm), illuminated from above with an LED light panel (59.5×59.5 cm, 48W Daylight; Opus Lighting Technology, Birmingham, UK). I allowed bees to enter the arena prior to the experiments and collected them for marking. Bees were individually marked using queen marking cages and unique paint colour combinations. During both the marking stages and the experiments, a nectar feeder was provided in the arena for bees to access *ad libitum*. Fresh flowers were collected in the morning of the experiments and individual flowers and placed in tubes filled with floral foam (OASIS Floral Products, Washington, UK). Flowers were presented at the front of the arena, near a mesh partition, on the other side of which was placed a digital recorder with two unidirectional microphones (Zoom Hn4 Pro Handy, Zoom North America, Hauppauge, NY). The recorder was consistently kept at 10cm from the flower. Audio recording is a well-established and effective method for capturing the frequency component of bee vibrations (De Luca et al., 2018).

4.3.4 Does buzzing change from S. citrullifolium to S. heterodoxum?

In this experiment, I allowed a naïve, individually marked bee from Colony 1 to forage freely for approximately 3 minutes (defined as a visit), while I recorded the audible component of her floral vibrations. Recordings were at made at 48kHz sampling rate for up to 3 minutes of floral buzzes. A visit was considered to have ended after a bee had buzzed for 3 minutes, or until she stopped buzzing (did not re-visit the flower after five minutes had passed). I removed any bee which was in the arena without initiating buzzing for 5 minutes. After a bee had finished or been removed, I repeated the same protocol for another marked bee. Fresh flowers were used for each bee in each visit. Individual bees were recorded on *S. citrullifolium* for six consecutive visits (n = 29 bees for the first visit and 18 bees for the sixth visits). Then, the same bees were exposed to *S. heterodoxum* for six additional visits and buzzes in the first (n = 10 bees) and sixth visits (n = 3 bees). The rapid drop-off in visitation success between visit 1 and 6 in *S. heterodoxum* reflects the reluctance of the bees to buzz and re-visit *S. heterodoxum*.

4.3.5 Does buzzing change from S. heterodoxum to S. citrullifolium?

Using the same protocol as above, I exposed naïve, marked bees from Colony 2 to *S. heterodoxum* for six visits and *S. citrullifolium* for a further six visits. This was performed to disentangle the effects of ageing/experience from flower type. Naïve bumblebees were less attracted to *S. heterodoxum* and reluctant to buzz and re-visit, possibly due to their small flowers: only 3 bees completed the first visits on *S. heterodoxum* and only 1 bee completed visits on both species.

After all experiments were completed, bees were euthanised by placing colonies in a -80°C freezer for 24 hours. I then measured the intertegular distance (ITD) of all identifiable marked bees using callipers. ITD is a commonly used estimate of body size in insects, including bees (Cane, 1987). Some bees could not be measured as their paint markings were no longer identifiable.

4.3.6 Data extraction

29 bees from Colony 1 performed one visit successfully on *S. citrullifolium*, and 18 of these performed six bouts on *S. citrullifolium*. 10 of these bees completed six bouts on *S. citrullifolium* and at least one visit on *S. heterodoxum*. 3 of these bees continued on to complete all 6 visits on *S. heterodoxum* (12 visits in total). Only 3 naïve bees from Colony

2 visited *S. heterodoxum* in the “reverse” experimental protocol. Consequently, recordings from the 10 bees from the “forward” experimental protocol are used for most of the further analyses and are referred to as the “focal bees”.

Recordings from individual focal bees in their first and sixth visits on each species were analysed using Audacity 2.1.2 (The Audacity Team, 2021). Each individual buzz was defined visually and aurally as each detectable segment of sound, at least 20ms apart. I obtained the frequency spectrum (using the Fast Fourier Transform in Audacity) of each individual floral buzz with a Hamming window (size = 512), and visually ascertained the peak frequency in Hz and the duration in milliseconds. I performed the same procedure for a single flight buzz from *every* bee’s first visit in *S. citrullifolium* (29 bees).

Recordings from all other visits (visits 2 to 5 from all bees on each species) were analysed using the *seewave* package in R v.4.0.2 (Sueur et al., 2008; R Development Core Team, 2021). Peak frequency was extracted from the entire recording using the *fpeaks* function. A high-pass filter of 250Hz was used in both techniques to filter out background noise and flight buzzes, which are significantly lower than floral buzzes (Pritchard and Vallejo-Marín, 2020).

4.3.7 Statistical analysis

To assess the relationship between size (ITD) and flight frequency, I used individual flight buzzes for 29 bees during their first visit to *S. citrullifolium* (1 buzz for each bee). I fitted a linear model, with flight frequency (Hz) as the response variable and size as an explanatory variable. To assess the effects of size and flight frequency on peak buzz frequency, I used the above flight frequencies and peak buzz frequencies for 29 bees during their first visit to *S. citrullifolium* (1 frequency value for each bee, extracted in R – see above). I fitted a linear model with floral buzz frequency (Hz) as the response variable and size and flight frequency as explanatory variables. as a fixed effect. To assess the relationship between size and buzz duration, I used individual buzzes from the first visit of the 10 focal bees to *S. citrullifolium*. I fitted a linear mixed model with duration (ms) as response variable, size (mm) as a fixed effect and bee id as a random effect.

To compare buzz characteristics between plant species, I compared the frequency and duration of individual buzzes in only the last visit to citrullifolium (n= bees, buzzes) and the first visit to heterodoxum (n= bees, buzzes). These visits were chosen to exclude confounding the effect of species with the effects of bee experience or bee age. I fitted linear mixed models with peak frequency (Hz) or duration (ms) or number of buzzes as the response, species as a fixed effect and bee identity as a random effect.

To assess the effect of experience within individual visits, I used the first sixty buzzes produced by each bee from visits 1 and 6 to each species as this represents the number of buzzes performed by most bees in each visit. I fitted separate linear mixed models for each visit, with peak frequency (Hz) or duration (ms) as the response, buzz number as a fixed effect and bee identity as a random effect.

To assess the effect of flower-handling experience over the course of visits to each species, I used the overall peak frequency of recordings from all 6 visits to each species, and the mean duration of buzzes from visits 1 and 6 to both species. I fitted linear mixed models with peak frequency (Hz) or duration (ms) as the response, visit number as a fixed effect and bee identity as a random effect. I also investigated the effect of visit number (visit 1 or 6) on the number of buzzes performed in *S. citrullifolium*. I fitted a linear model with number of buzzes as the response and visit number as the explanatory variable. I could not assess this effect in *S. heterodoxum*, as too few bees continued on to visit 6.

Model diagnostics were performed using the package *DHARMa*. Models were fitted in the *stats* and *lme4* package. Statistical significance of the main effects were assessed using Type II sums of squares in the package *car*. All statistical analysis was performed in R 4.0.2 (R Core Team 2021).

4.3.8 Ethics

Bumblebee experiments were approved by the Animal Welfare and Ethical Review Board at the University of Stirling (Appendix A).

4.4 Results

4.4.1 Does bumblebee body size affect flight frequency or buzz characteristics?

Recorded bees of Colony 1 varied in size from a minimum ITD of 4mm and a maximum of 5.1, with a mean of 4.71±0.05 (std. error) (n = 29 bees which were recorded in an initial visit to *S. citrullifolium*). The 10 focal bees (those for which individual buzzes were analysed in visits 1 and 6 on both plant species) varied in ITD from 4.05 to 4.9mm, with a mean of 4.61±0.09. Flight frequency of the 29 bees varied from 144 to 180Hz, with a mean of 163.52±1.56. Flight frequency of the 10 focal bees varied from 144 to 174Hz, with a mean of 162.2±3.23 Hz. In the 29 bees, I found no effect of bee size on flight frequency (Table 4.1A, Figure 4.2A) or of bee size or flight frequency on buzz frequency (Table 4.1B, Figure 4.2B and D). There is also no effect of size on buzz duration in the 10 focal bees (Table 4.1C, Figure 4.2C).

	Estimate	**Std. error**	***P*-value**
A. Flight frequency			
Size	-3.43	5.61	0.53
B. Buzz frequency			
Size	-0.06	0.03	0.06
Flight frequency	-0.00	0.00	0.38
C. Duration			
Size	-152.72	76.26	0.09

Table 4.1 A: Linear model fitted with flight frequency (Hz) as response variable and bee size (ITD, mm) as explanatory variable. B: A: Linear model fitted with buzz frequency (Hz) as response, bee size (mm) and flight frequency (Hz) as explanatory variables. C: Linear mixed model with duration (ms) as response variable, size (mm) as a fixed effect and bee id as a random effect. Sample sizes: A and B: 29, C: 10.

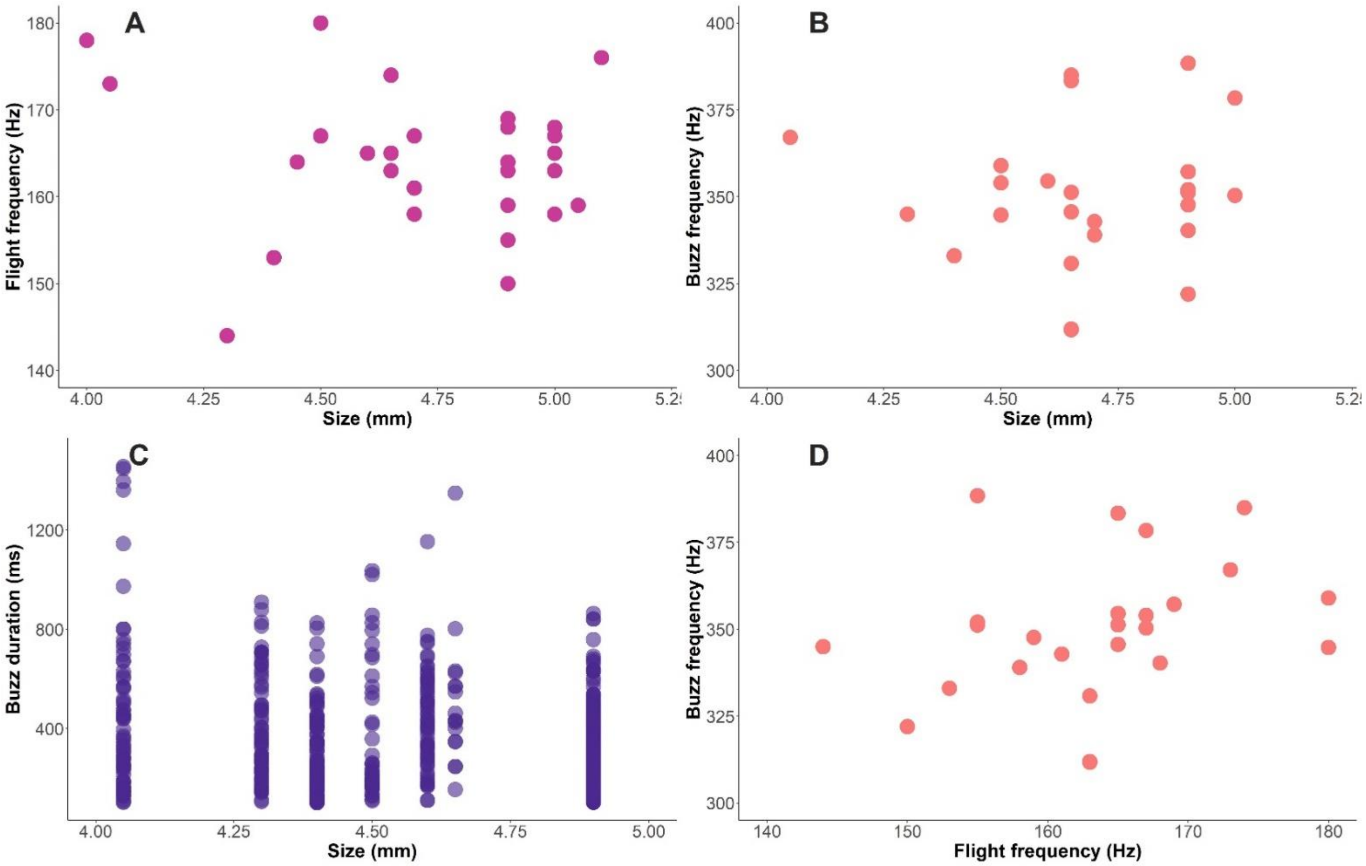


Figure 4.2 Bee size (ITD in mm) against flight frequency (Hz) (A). Bee size (mm) against floral buzz frequency (Hz) (B). Bee size (mm) against floral buzz duration (ms) (C). Flight frequency (Hz) against floral buzz frequency (Hz) (D). Data are for 29 marked bees in their first visit to *S. citrullifolium.*

4.4.2 Does plant species affect buzz characteristics?

I compared individual buzzes from 10 focal bees in their last visit to *S. citrullifolium* (485 buzzes) and their first visit to *S. heterodoxum* (283 buzzes). The mean number of buzzes performed by bees on *S. citrullifolium* was 100.4±15.1 (range: 45 to 191), while the mean buzz number on *S. heterodoxum* was 58.6±9.7 (range: 21 to 127). Mean peak buzz frequency (Hz) on *S. citrullifolium* was 343.86±0.57 (range: 280 to 406) and on *S. heterodoxum* was 349.6±0.68 (range: 265 to 392). I found a small but statistically significant effect of plant species on buzz peak frequency (Hz, coefficient: 3.98, $p<0.001$, $n=768$), with bees buzzing at slightly higher frequencies on *S. heterodoxum* than on *S. citrullifolium* (Table 4.2A, Figure 4.3).

Mean buzz duration (ms) on *S. citrullifolium* was 307.42±5.98 (range: 101 to 1531) and on *S. heterodoxum* was 267.22±6.68 (range: 101 to 1328). I found a small effect of species on buzz duration (ms, coefficient: -49.36, $p<0.001$, $n=743$) with shorter buzzes performed on *S. heterodoxum* than on *S. citrullifolium* (Table 4.2B, Figure 4.3).

I found an effect of species on buzz number (coefficient: -41.8, $p<0.05$, $n=20$), with bees performing more buzzes in their last visits to *S. citrullifolium* compared to their first visit to *S. heterodoxum* (Table 4.2C, Supplementary Figure 4.6).

	Estimate	**Std. error**	***P*-value**
A. Peak frequency			
Species	3.98	1.14	**<0.001**
B. Duration			
Species	-49.36	13.14	**<0.001**
C. No. buzzes			
Species	-41.8	76.26	**<0.05**

Table 4.2 Linear mixed models fitted with peak frequency (Hz, A), duration (ms, B) or number of buzzes as the response variable, plant species as a fixed effect and bee id as a random effect. Sample sizes: A: 768; B: 743; C: 20.

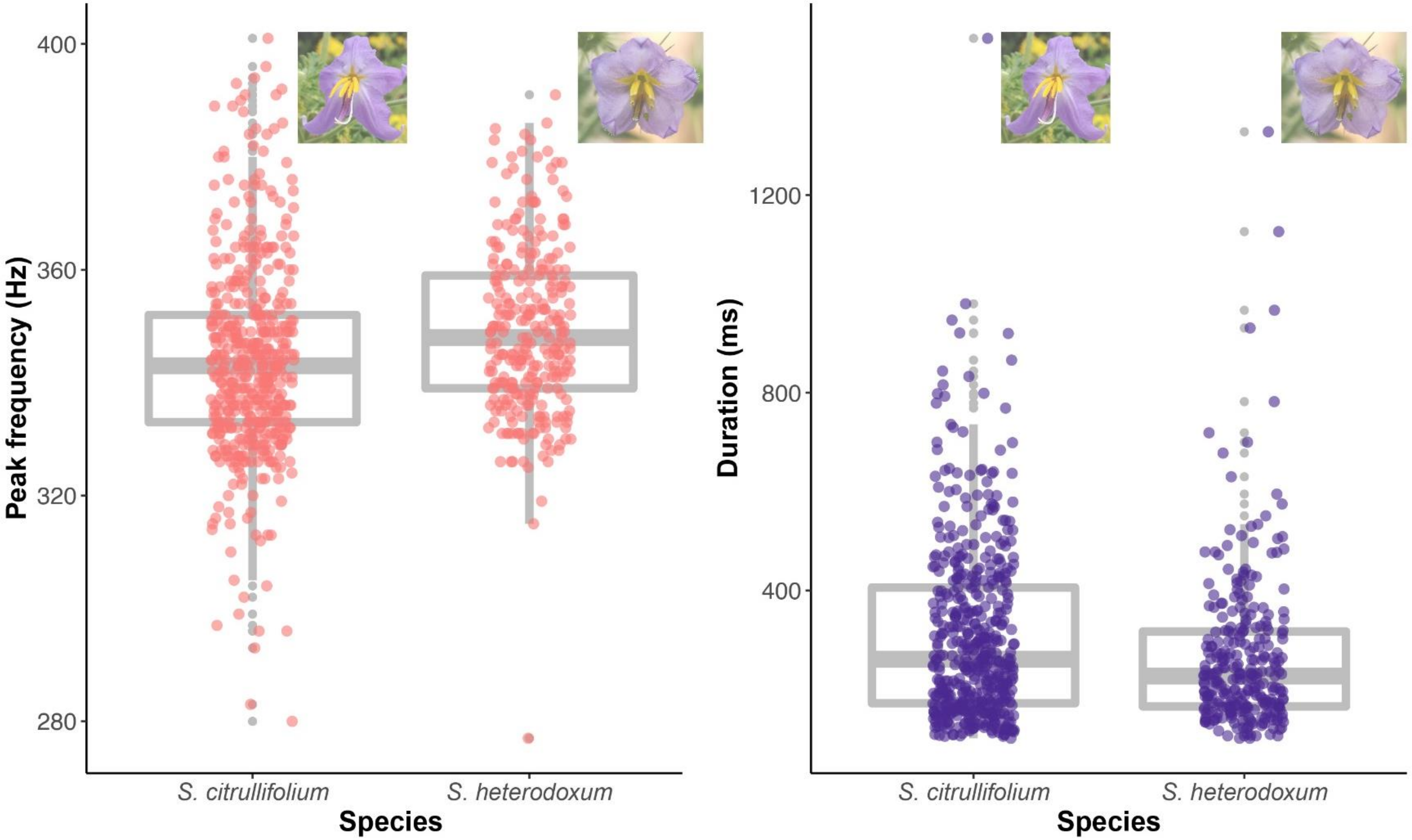


Figure 4.3 Comparison of individual buzz characteristics of 10 focal bees between visit 6 on *S. citrullifolium* (485 buzzes) and visit 1 on *S. heterodoxum* (283 buzzes). Species v peak frequency (Hz) (left) and species v duration (ms) (right).

4.4.3 Do bee buzzes change as they gain handling experience within visits?

I found no effect of buzz number (over sixty buzzes) on buzz peak frequency (Hz) or duration (ms) within three of the four analysed visits to both species by 10 bees: buzz characteristics within a visit do not change (Figure 4.4.3). Within the first visit to *S. heterodoxum*, however, there is a slight but statistically significant increase in buzz peak frequency (Hz) throughout the visit (coefficient: 0.12, p<0.01, n=456, Table 4.3C, Figure 4.4E). See Supplementary Figure 4.7 for all buzzes performed by individual bees over each of the analysed visits.

	Estimate	**Std. error**	***P*-value**
A. Frequency – visit 1 (cit)			
Buzz number	-0.08	0.04	0.06
B. Frequency – visit 6 (cit)			
Buzz number	0.14	0.04	0.25
C. Frequency – visit 1 (het)			
Buzz number	0.12	0.04	**<0.01**
D. Frequency – visit 6 (het)			
Buzz number	0.03	0.05	0.56
E. Duration – visit 1 (cit)			
Buzz number	1.22	0.64	0.06
F. Duration – visit 6 (cit)			
Buzz number	-0.08	0.49	0.87
G. Duration – visit 1 (het)			
Buzz number	-0.5	0.44	0.26
H. Duration – visit 6 (het)			
Buzz number	0.46	0.84	0.59

Table 4.3 Linear mixed models for each visit, fitted with peak frequency (Hz) or duration (ms) as the response variable, buzz number as a fixed effect and bee id as a random effect. Sample sizes: A and E: 467; B and F: 548; C and G: 456; D and F: 114.

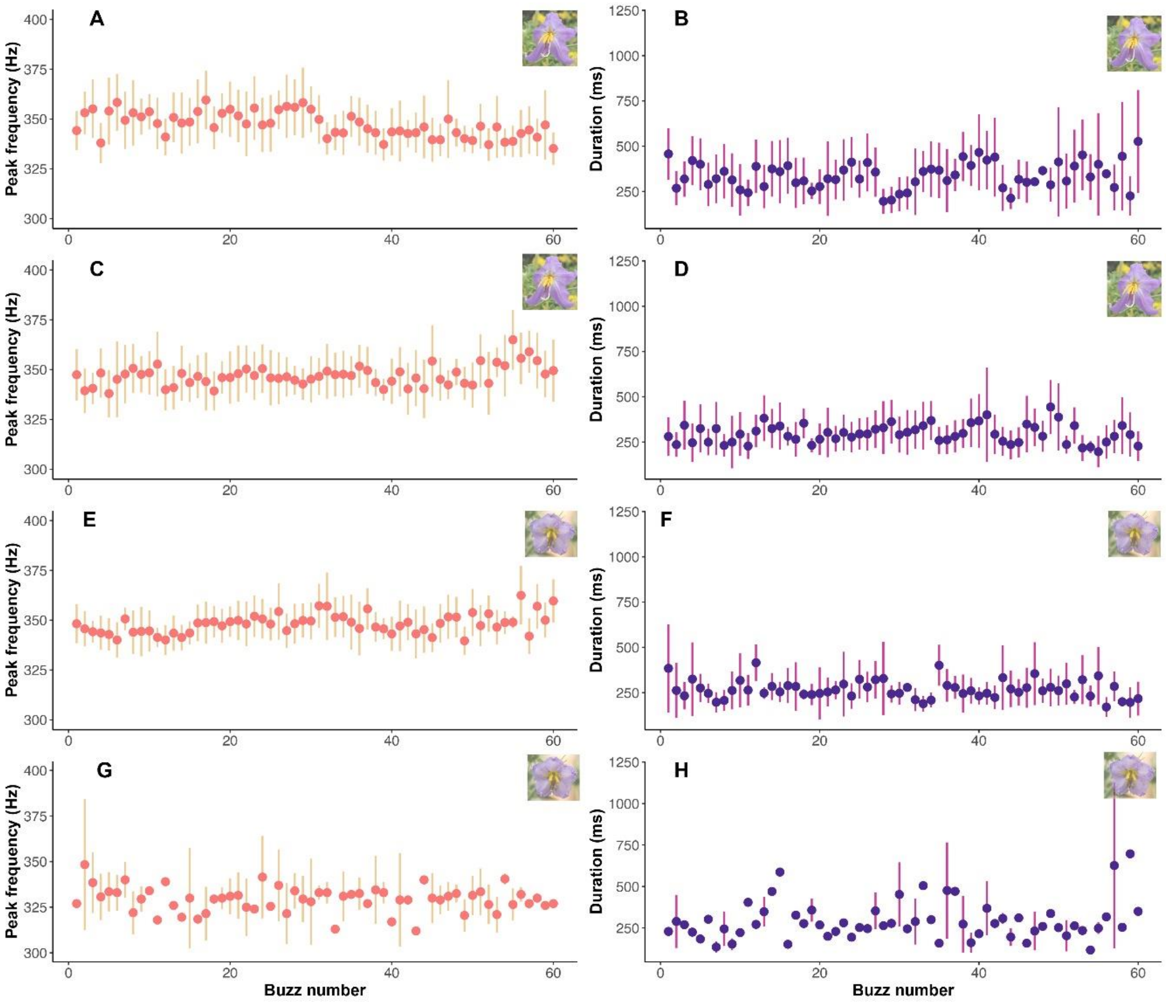


Figure 4.4 Buzz number against buzz characteristics for the first 60 buzzes by 10 focal bees in each analysed visit: peak frequency (Hz) (left) and duration (ms) (right). Visit 1 on *S. citrullifolium* (A and B); visit 6 on *S. citrullifolium* (C and D); visit 1 on *S. heterodoxum* (E and F); visit 6 on *S. heterodoxum* (G and H).

4.4.4 Do bee buzzes change as they gain experience across successive visits?

I found no effect of visit number on buzz peak frequency (Hz) across all 6 visits in either *S. citrullifolium* (n=145, 29 bees, Table 4.4A, Figure 4.5A) or *S. heterodoxum* (n=29, 10 bees, Table 4.4B, Figure 4.5C) . I also found no effect of visit number (1 or 6) on buzz duration in either *S. citrullifolium* (n=20, Table 4.4C, Figure 4.5B) or *S. heterodoxum* (n=13, Table 4.4D, Figure 4.5D). The number of buzzes changed significantly between visits 1 and 6 on *S. citrullifolium* (coefficient: 36.8, p<0.05, n=20, Table 4.4E, Supplementary Figure 4.6), with bees performing more buzzes in their 6th visit to this species.

	Estimate	**Std. error**	***P*-value**
A. Frequency – *S. citrullifolium*			
Visit number	-0.003	0.001	0.05
B. Frequency – *S. heterodoxum*			
Visit number	8.96E-4	1.1E-3	0.42
C. Duration – *S. citrullifolium*			
Visit number	-34.8	29.72	0.27
D. Duration – *S. heterodoxum*			
Visit number	-37.25	30.29	0.32
E. Number of buzzes – *S. citrullifolium*			
Visit number	36.8	17.42	0.05

Table 4.4 A-D: Linear mixed models for each species, fitted with peak frequency (Hz) or duration (ms) as the response variable, visit number as a fixed effect and bee id as a random effect. E: Linear model fitted for *S. citrullifolium*, with number of buzzes as the response and visit number (one or six) as the explanatory variable. Sample sizes: A: 145 ; B: 29, C: 20; D:13; E: 20.

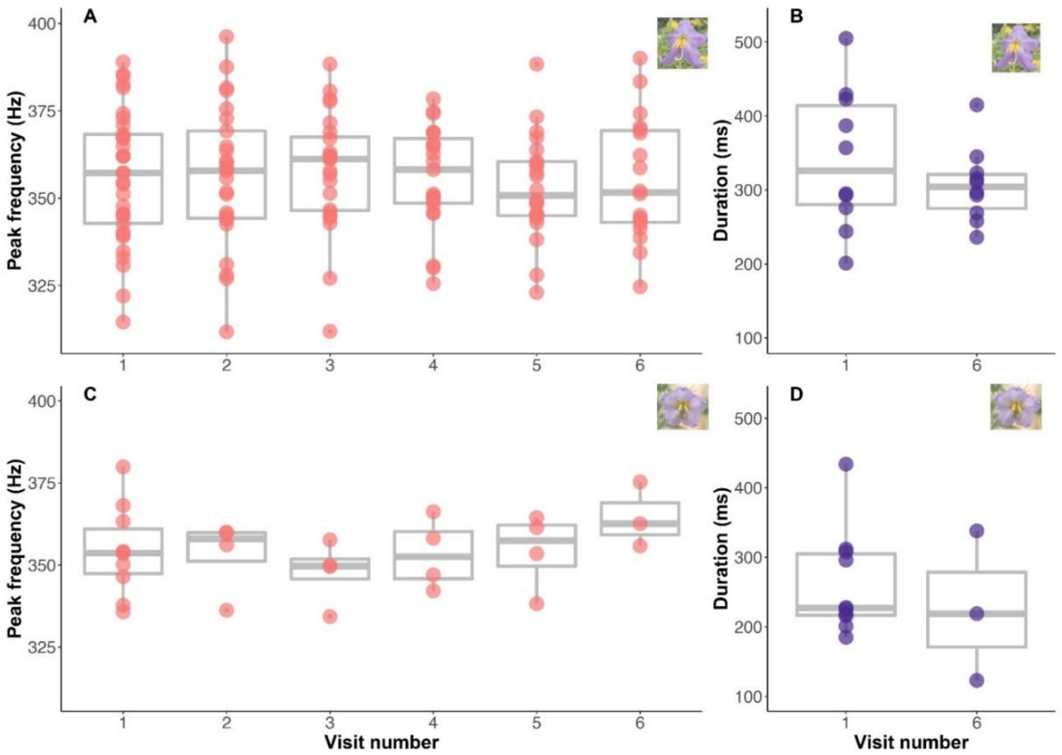


Figure 4.5 Visit number against buzz frequency (Hz) on 6 consecutive visits to *S. citrullifolium* in 29 bees (A). Visit number against buzz duration (ms) in analysed visits 1 and 6 to *S. citrullifolium* in 10 focal bees (B). Visit number against buzz frequency (Hz) on 6 consecutive visits to *S. heterodoxum* in 10 focal bees (C). Visit number against buzz duration (ms) in analysed visits 1 and 6 to *S. heterodoxum* in 10 focal bees (D). The low number of data points in visits 2 to 6, panels C and D, reflects the drop-off in bee visitation rates to *S. heterodoxum* after visit 1.

4.5 Discussion

Floral vibrations are costly to produce, and bees should be motivated to minimise these costs whilst maximising the amount of pollen collected (Russell et al., 2017). One strategy could be to match their vibration frequency to stamen natural frequencies, thus amplifying their vibrations and potentially increasing pollen release (King and Buchmann, 1996; Nunes et al., 2021). Buzz-pollinated flowers vary in their stamen natural frequency (Nunes et al., 2021), implying that bees might adjust their vibration frequency when handling different species. Here I investigated bumblebee buzz characteristics on two species with different stamen natural frequencies, as well as different sizes, morphologies, and pollen release rates. Bee vibrations do not match the stamen natural frequency in either species, but small differences in buzz behaviour may indicate a response to pollen availability and release rates.

I found no relationship between body size (ITD) and buzz frequency, either in flight buzzes or floral buzzes. Within insects, flight (wingbeat) frequency is negatively associated with body size, based on both biomechanical principles and empirical evidence from a range of bee species (Corbet and Huang, 2014; De Luca et al., 2019) and across other insect taxa: larger insects have lower wingbeat frequencies than smaller insects (Molloy et al., 1987; Deora et al., 2017). Within bumblebee species, there is some evidence of this effect (Corbet and Huang, 2014), although the relationship is somewhat weaker than across insects generally (Pritchard and Vallejo-Marín, 2020). With a range of intertegular distances of only around 1mm, the bumblebees used in this experiment may not provide a wide enough range of body sizes for any such effect to be apparent.

The peak frequency of floral buzzes is higher than flight buzzes across bee families (Corbet et al., 1988; King and Lengoc, 1993; Harder and Barclay, 1994), but the relationship between bee size and floral buzz frequency is complex. The ratio between flight frequency and floral buzz frequency apparently changes with size across diverse bee taxa: larger bees have lower flight frequencies but raise their floral buzz frequencies to a greater extent than smaller bees, flattening any potential relationship between size and floral buzz frequency (Burkart et al., 2011; De Luca et al., 2019). This may explain why some studies fail to find a relationship between floral frequency and size (Rosi-Denadai et al., 2020). Although this ‘buzz ratio’ effect may be partly driven by behavioural or cognitive factors which vary between taxa and could be confounded with size, Switzer et al. (2019) found that within *Bombus impatiens*, larger bees have lower floral buzz frequencies and have more flexibility in their buzz characteristics. Larger-bodied bees increase both their frequency and amplitude in response to pollen feedback, whilst smaller bees may be physiologically limited to a smaller range of buzz characteristics (Switzer et al., 2019). This difference in flexibility has been little explored, but a negative association between size and buzz frequency has been found in other bumblebee species (Corbet and Huang, 2014; Pritchard and Vallejo-Marín, 2020). However, other studies, including this present study, have found no evidence of a relationship (De Luca et al., 2013; Nunes-Silva et al., 2013; De Luca et al., 2014).

I also found no effect of body size on individual buzz duration in the focal bees. De Luca et al. (2014) found a negative association across three bumblebee species, with larger bees producing shorter buzzes. Buzz duration has been less explored than frequency and is highly variable within and between bumblebee individuals, likely influenced by several factors, including a bee’s energy reserves. It is perhaps the most plastic property of bee buzzes, and most within the capacity of the bee to control, but may interact with body size. For instance, it may be most efficient, both in time and energy, for large bees to produce a short, higher-amplitude buzz, perhaps releasing the same amount of pollen as a long, lower-amplitude buzz produced by a small bee (Switzer et al., 2019).

The clearest effect of plant species on bee behaviour in this study is that both naïve and experienced foragers are reluctant to visit *S. heterodoxum*, despite the absence of any foraging alternatives. The reluctance of naïve bees may reflect a lack of attractiveness of *S. heterodoxum*, perhaps due to its small flower size or floral volatiles, which vary between these species (Vallejo-Marín et al., 2014) (Moore, unpublished). The evolution of small flower size is associated with an increase in selfing across several *Solanum* taxa, including these species (Tang and Huang, 2007; Vallejo-Marín et al., 2014). *S. citrullifolium* is dependent on pollinators for outcrossing and its size and scent likely promote high visitation rates (Vallejo-Marín et al., 2014). Visual and scent cues in *S. heterodoxum* may not be sufficiently salient to entice visitation by naïve bees (Katzenberger et al., 2013; Orbán and Plowright, 2014).

Many of the bees experienced on *S. citrullifolium* first also rejected *S. heterodoxum* in their first encounter. This may not be wholly related to the salience of *S. heterodoxum* cues, as flowers were positioned in the same location in the arena across all visits, and bees were accustomed to visiting this point for foraging and spatial memory plays an important role in bumblebee foraging behaviour (Saleh and Chittka, 2007). Spatial memory may thus negate differences in salience, although volatiles detected at close proximity to the flower may still play a role in rejection. Instead, bees likely learned through the prior six visits to associate the large flower size (or scent) of *S. citrullifolium* with rewards, perhaps making them more likely to reject flowers which differ from this template (Blarer et al., 2002; Muth et al., 2016). Of the bees who visited *S. heterodoxum* in their first encounter, most declined to re-visit in subsequent attempts. This suggests that *S. heterodoxum* is not sufficiently rewarding for bees to spend time and effort visiting, which may be reinforced by their memory of the large flower as highly rewarding (Buchmann and Cane, 1989; Blarer et al., 2002). This effect may be related to differences in pollen availability and release between species, as will be discussed later in this discussion.

Evidence that bees adjust their buzz frequencies to different plant species is minimal and somewhat conflicting. Here, I tested this on species with known differences in stamen natural frequency. I find that bees do not tune their frequencies to each species. Floral vibrations are on average 343Hz on *S. citrullifolium* and 349Hz on *S. heterodoxum*. The natural frequencies of stamens of *S. citrullifolium* are below 100Hz, whilst those of *S. heterodoxum* are between 100-150Hz (Chapter Three) (Nunes et al., 2021). Furthermore, if they were attempting to match the stamen natural frequency, we might expect them to lower their frequency over time, both in the first visit to *S. citrullifolium* and the first visit to *S. heterodoxum*, but we see no such effect. Similarly, a previous study similarly found no change in buzz frequency within bumblebee visits on *S. lacianiatum* (King and Buchmann, 1996). The natural frequencies in this species and the *Solanum* flowers used here are likely too far outside the range of bumblebee vibrations for this to be a strategy used here. However, evidence using artificially dispensed pollen suggests that bees do not tune to an optimal frequency even if it is within reach (Switzer et al., 2019).

This study has only investigated one bee species visiting two related plants. In nature, visitors to buzz-pollinated plants vary substantially in body size (Mesquita-Neto et al., 2018; De Luca et al., 2019; Mesquita-Neto et al., 2021). Likewise, we expect a wide range in natural frequency amongst buzz-pollinated plants, given the substantial variation in floral morphologies. This creates an opportunity for natural frequency to be exploited in specific contexts. For instance, small-bodied bees may have a lot to gain from matching the natural frequency of flowers – they may not be able to produce as much energy as large bees (De Luca et al., 2013; De Luca et al., 2014), and might compensate by changing their buzz frequency. This may be particularly advantageous on flowers with relatively high stamen natural frequency, e.g. flowers with short or stiff stamens. On the other hand, large bees may be more flexible in their buzzing behaviour (Switzer et al., 2019) and already produce relatively low floral vibrations (Burkart et al., 2011; De Luca et al., 2019). As these vibrations are closer in frequency to the stamen natural frequencies so far measured (Nunes et al., 2021), these large bees may be in a better position to exploit this trait. Lastly, even if bees do not actively employ this strategy, frequency-matching may inadvertently occur, given the variation in bee and flower traits. This may have implications for floral trait evolution, via the function of poricidal anthers as pollen dispensers.

Bees may alter their behaviour between plant species as a response to differences in pollen release and availability. My results suggest several lines of evidence to support this hypothesis. Broadly, total pollen availability varies between these species (Kemp and Vallejo-Marín, 2021), and the relatively low amount of pollen collected per visit on the smaller species may have affected bee visitation rates, discouraging most bees from visiting *S. heterodoxum* a second time. Bees also spent less time buzzing the small *S. heterodoxum* flowers, both in number of buzzes performed and the duration of each buzz. The drop-off in buzzes during visits may be explained by diminishing pollen returns from the flower – *S. heterodoxum* releases pollen at a faster rate than *S. citrullifolium*, and after 50 or so buzzes, bees may be collecting very little (Kemp and Vallejo-Marín, 2021). There is substantial evidence that bees spend less time handling unrewarding flowers and are faster to give up foraging on them (Buchmann and Cane, 1989; Russell et al., 2016b; Switzer et al., 2019), although many studies compare rewarding flowers with those completely lacking rewards. Buchmann and Cane found that *Bombus sonorus* and *Ptiloglossa arizonensis* both perform fewer buzzes and shorter visits on *Solanum* flowers with experimentally depleted pollen (Buchmann and Cane, 1989), although bees had the option to visit more rewarding flowers instead, unlike in this present study. Likewise, nectar-foraging bees also reduce their visits to flowers when nectar rewards are low (Manetas and Petropoulou, 2000; Dreisig, 2012).

Differences in buzz duration may also be related to pollen feedback. Russell et al. (2016b) found that *B. impatiens* individuals increased buzz length within a visit to large, heterantherous *S. houstonii* flowers, which are comparable to *S. citrullifolium* in both size and morphology. It is possible that short buzzes on *S. heterodoxum* may release a large proportion of pollen, compared to equivalent buzzes on *S. citrullifolium*, although this has not been investigated. Alternatively, bees may be primed from their prior experiences to expend less energy on *S. heterodoxum*, perhaps to as a form of bet-hedging.

Intriguingly, bees buzzed at slightly higher frequencies on *S. heterodoxum* than *S. citrullifolium*. The difference in frequency here is small (~6Hz) and unlikely to substantially affect pollen release but may be a response to differences in pollen feedback. Bumblebees have been shown to buzz at higher frequencies when unable to access pollen (Russell et al., 2016b; Switzer et al., 2019). This may explain the increase in buzz frequency during the first visit to *S. heterodoxum*, as the flower released less pollen throughout the visit.

One caveat to the pollen release hypothesis is that I did not measure pollen collection during the bees' visits to different species. Future experiments could corroborate the suggested effect of pollen feedback on bee behaviour by measuring foraging efficiency, for instance in a study of bee buzzing behaviour on flowers with artificially altered pollen dispensing curves. Likewise, it would be enlightening to investigate the response of bees to different flower types when there is a range of options to choose from as this is likely to affect the trade-offs involved in foraging decisions.

These results demonstrate some flexibility in buzzing behaviour but suggest that bees are not fine-tuning their buzz frequencies to each flower type. I propose that bees are responding somewhat to pollen release rates and total pollen availability, mostly in terms of overall foraging effort. The apparent flexibility of bee buzzing, leading to much of the variation seen within individuals, likely consists of specific responses to particular circumstances, i.e. behavioural rules: when pollen is absent, raise the frequency of the buzz, or: when pollen is plentiful, buzz more and for longer. In this sense, variation in buzz properties *is* linked to plant species, but this is mediated by pollen release rather than floral morphology *per se*.

4.6 Supplementary material

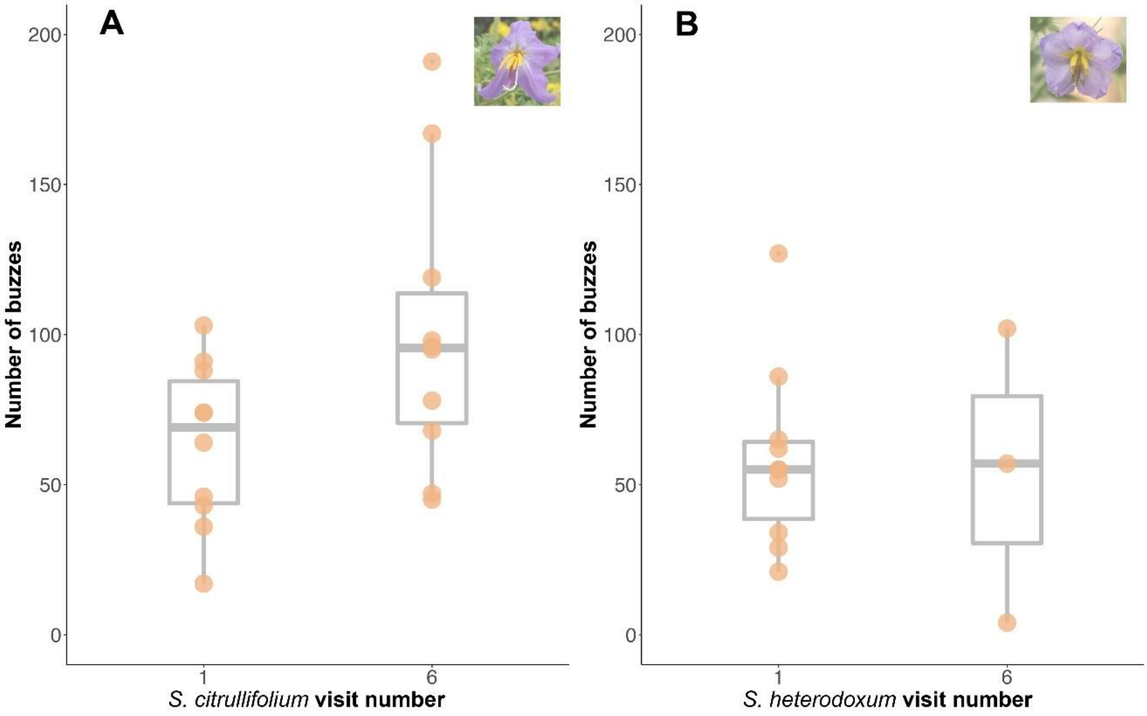


Figure 4.6 Number of buzzes performed in each analysed visit for 10 focal bees on each flower type (visits 1 and 6 in S. citrullifolium and S. heterodoxum).

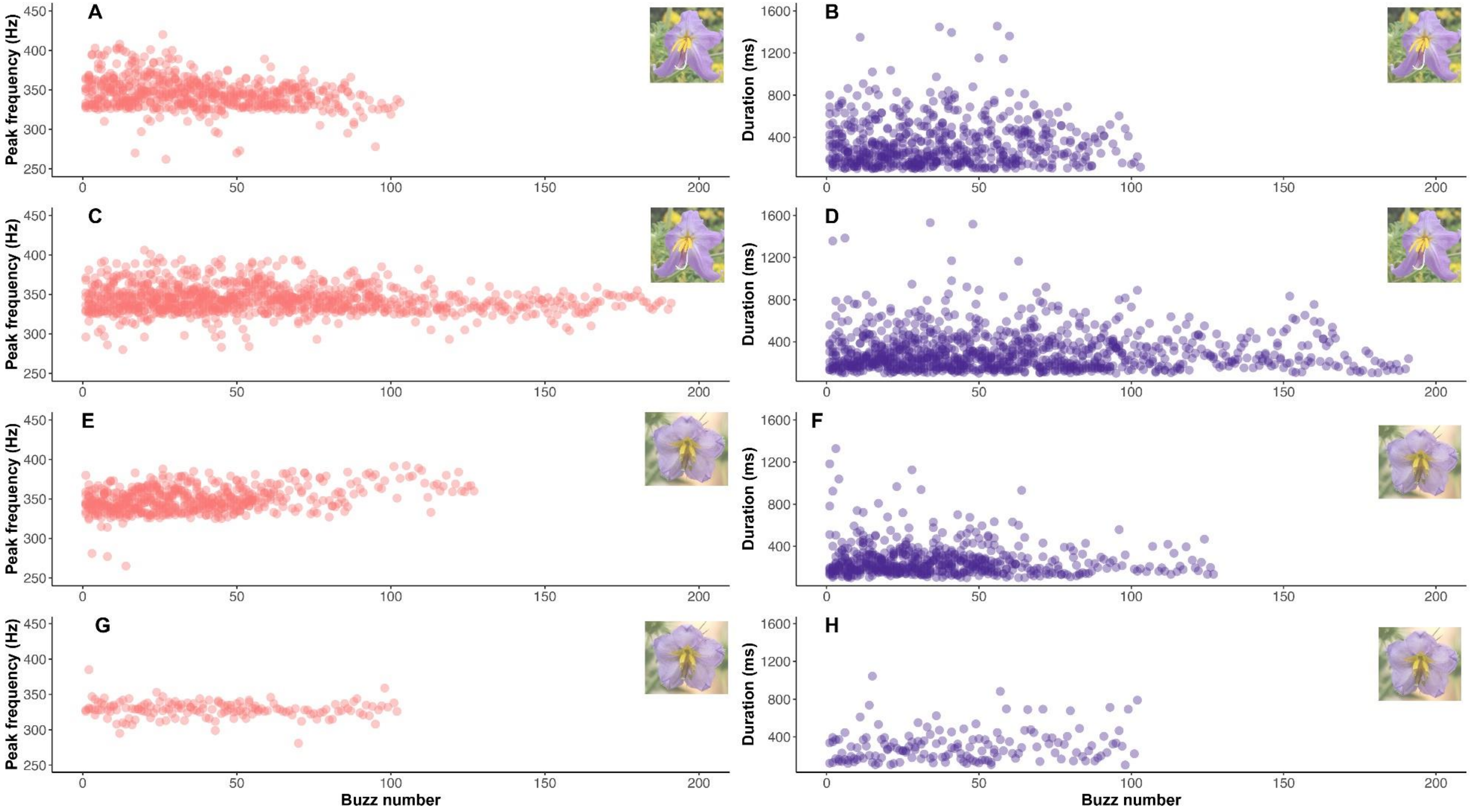


Figure 4.7 Buzz number against buzz characteristics for all buzzes from 10 focal bees in each analysed visit: peak frequency (Hz) (left) and duration (ms) (right). Visit 1 on *S. citrullifolium* (A and B); visit 6 on *S. citrullifolium* (C and D); visit 1 on *S. heterodoxum* (E and F); visit 6 on *S. heterodoxum* (G and H).

Chapter Five: Floral orientation affects pollen deposition in buzz-pollinated flowers with bilateral symmetry

5.1 Abstract

Floral orientation is central to plant-pollinator interactions and is commonly associated with floral symmetry. Bilaterally symmetrical flowers are often oriented horizontally for optimal pollinator positioning and pollen transfer efficiency, while the orientation of radially symmetrical flowers is variable. Buzz-pollinated flowers display a range of morphologies and orientations, including bilateral, horizontally oriented flowers and radial, pendant flowers. The effect of orientation on pollen transfer may differ between radial and bilateral flowers, particularly those which possess more than one stamen type. Here, we examine the effect of floral orientation on bumblebee-mediated pollen deposition in three *Solanum* species with different natural orientations: *S. lycopersicum* and *S. seaforthianum* (radial, pendant), and *S. rostratum* (bilateral, horizontal). We first ask whether orientation (pendant or horizontal) affects total stigmatic pollen deposition (both self and outcross pollen). We find no effect of floral orientation on pollen deposition across the three species. We then ask whether a mismatch between donor and recipient orientation affects outcross pollen deposition in *S. rostratum.* We find that these bilaterally symmetrical flowers receive less pollen when they are pendant recipients or have pendant donors, compared to flowers which are horizontal recipients or have horizontal donors. Our results support the hypothesis that bilaterally symmetrical, buzz-pollinated flowers are oriented horizontally to increase cross pollination.

5.2 Introduction

Floral orientation, the position of the corolla's main axis relative to the horizontal, is a key trait mediating plant-pollinator interactions (Stebbins, 1974; Fenster et al., 2009; Armbruster and Muchhala, 2020). Across angiosperms, floral orientation is widely associated with floral symmetry: bilaterally symmetrical flowers are often oriented horizontally to promote precise pollinator positioning (Giurfa et al., 1999; Fenster et al., 2009; Armbruster and Muchhala, 2020). Buzz-pollinated plants rely on bee vibrations for pollen release, and bees are expected to adopt positions on these flowers that are most effective for buzzing, which can be highly constrained by floral symmetry and stamen arrangement (Buchmann, 1983; Russell et al., 2017). Buzz-pollinated flowers may therefore have a particularly high requirement for precise pollinator positioning, along with a concomitant requirement from the bee for the most efficient buzzing position, compared to flowers in which pollen is collected by "scrabbling" or other methods (Russell et al., 2017; Portman et al., 2019). The relationship between orientation and floral symmetry is therefore expected to play an important role in pollen transfer in these plants (Neal et al., 1998; Armbruster and Muchhala, 2020). Here, we investigate the effects of these floral traits on bumblebee-mediated pollen transfer in *Solanum* spp., which vary in both floral orientation and floral symmetry.

The drivers of floral orientation have primarily been investigated in non-buzz pollinated species but are likely to differ somewhat in buzz pollinated plants. In many flowers, a pendant, i.e. downward facing, orientation protects pollen and nectar from the elements, for example rainfall (Huang et al., 2002; Aizen, 2003; Wang et al., 2010; Lin and Forrest, 2017), or high temperatures (Haverkamp et al., 2019). However, most buzz-pollinated flowers are nectarless and have poricidal anthers, i.e., the pollen is contained inside the anther and released through small apical pores (Harder and Barclay, 1994). Pollen is thus relatively protected, suggesting that this is not an important driver of a pendant orientation, although it is possible that exposure to the elements could affect the microclimate inside the anther (Corbet et al., 1988). For example, humidity may affect pollen release, and the anthers of vertical flowers may be more dehydrated than in pendant flowers, due to exposure to wind and sun (Corbet et al., 1988), although this has yet to be thoroughly investigated. Orientation can also be associated with pollinator groups. For instance, lepidopterans and syrphid flies can handle vertical flowers more easily than pendant flowers (Ushimaru et al., 2009; Makino and Thomson, 2012; Campbell et al., 2016; Haverkamp et al., 2019; Mei Ying et al., 2020; Xiang et al., 2021), while many birds prefer to visit pendant flowers (Aizen, 2003; Xiang et al., 2021), and a shift from hawkmoth to hummingbird pollination in *Loricera spp.* is associated with a transition from vertical to pendant flowers (Xiang et al., 2021). In buzz-pollinated plants, only bees (Anthophila) can release pollen via floral vibrations (Vallejo-Marín and Vallejo, 2021), but for possible exceptions see Buchmann (1977). This suggests that orientation might have other roles within this broad pollinator group (bees). Bumblebees (*Bombus spp.*) tend to prefer vertical to pendant flowers (Makino and Thomson, 2012), even when flowers are naturally pendant (Prokop et al., 2020), but they will readily visit all orientations and can handle pendant flowers more effectively than other insects (Wang et al., 2014a; Haverkamp et al., 2019; Prokop et al., 2020). Buzz-pollinated pendant flowers could benefit from excluding non-bee visitors as has been shown in nectar-rewarding, not buzz-pollinated species where a pendant orientation in *Geranium refractum* excludes ineffective non-bee visitors (Wang et al., 2014b; Prokop et al., 2020).

Crucially, the effect of floral orientation on pollination is often related to the symmetry of the flower. Zygomorphic or bilaterally symmetrical flowers require more precise pollinator positioning than actinomorphic or radially symmetrical flowers (Neal et al., 1998; Giurfa et al., 1999; Wang et al., 2014a; Armbruster and Muchhala, 2020) and have fewer potential pollinators (Yoder et al., 2020). According to the “pollen position hypothesis” (Neal et al., 1998), bilateral flowers constrain the position of the bee on the flower. This promotes consistency in the site of contact between the pollinator body and the flower’s sexual organs, impacting both pollen removal and deposition (Neal et al., 1998; Giurfa et al., 1999; Ushimaru et al., 2009; Armbruster et al., 2014; Culbert and Forrest, 2016; Armbruster and Muchhala, 2020). A horizontal orientation further restricts pollinator positioning, limiting the angles from which a pollinator can approach the flower, as shown in honeybees visiting *Roepera* species (Fenster et al., 2009; Naghiloo et al., 2020). Additionally, there is a strong association between bilateral symmetry and horizontal orientation across animal-pollinated plants (Giurfa et al., 1999; Ushimaru et al., 2009; Naghiloo et al., 2020) and bilateral flowers vary less in orientation between and within plants (Giurfa et al., 1999). This holds true even when the plant’s pollinators are primarily associated with non-horizontal flowers, such as the hawkmoth-pollinated *Abelia x grandiflora* (Mei Ying et al., 2020) or the syrphid-pollinated *Commelina communis* (Ushimaru and Hyodo, 2005; Ushimaru et al., 2009). Some bilateral flowers also restore their correct orientations after being experimentally manipulated, unlike radial flowers, demonstrating that floral symmetry mediates selection on specific orientations (Armbruster and Muchhala, 2020).

Solanum (Solanaceae) is ideally suited for investigating the effect of floral orientation on the function of buzz-pollinated flowers. *Solanum* contains around 1400 species (Särkinen et al., 2013), with floral orientations ranging from pendant (*Solanum dulcamara*, *S. lycopersicum*) to horizontal (*S. citrullifolium*, *S. rostratum*) (Vallejo-Marín et al., 2010) and occasionally almost vertical (*S. houstonii*)(Knapp et al., 2017). *Solanum* species also differ in floral symmetry. Most flowers in the genus are radially symmetrical, with equally sized anthers arranged in a cone surrounding the style, and petals often reflexed (Endress, 1996; Glover et al., 2004; Bohs et al., 2007). This "solanoid" cone flower has evolved many times in other buzz-pollinated lineages, such as Melastomataceae and Primulaceae (Faegri, 1986). *Solanum* also contains zygomorphic species, notably those which possess at least two stamen morphs (heterantherous) (Bohs et al., 2007; Vallejo-Marín et al., 2010). Heteranthery has evolved at least four times across the clades of *Solanum* (Bohs et al., 2007; Vallejo-Marin et al., 2009; Vallejo-Marín et al., 2010), and a loss in heteranthery is associated with a switch in reproductive strategy from outcrossing to selfing in some lineages (Vallejo-Marín et al., 2010). Effective outcross pollen transfer relies on bees contacting both the anther and the stigma (Solís-Montero and Vallejo-Marín, 2017). Pollinator positioning is key and is likely aided by a horizontal orientation restricting the angle from which the bee can approach and land. Flowers with marked heteranthery, such as those seen in section Androceras, are usually horizontally or vertically oriented, while radially symmetrical flowers with anther cones are often pendant or between pendant and horizontal (Consortium). A possible exception to this is *Solanum thelopodium*, which is largely pendant but technically heterantherous (Consortium; Bohs et al., 2007), although less strongly zygomorphic than other heterantherous *Solanum* species. If floral orientation in heterantherous flowers affects the approach and landing position of bees, thereby altering the point of contact with both the anther and stigma, this may have consequences for pollen transfer. Radially symmetrical, cone-shaped flowers require less specific pollinator positioning and pollen transfer may be less affected by manipulations of orientation. This variation in orientation, symmetry and mating strategies across the *Solanum* genus offers a valuable system in which to examine the relationship between floral orientation and pollen transfer in buzz-pollinated plants.

Here, we investigate the effect of floral orientation on stigmatic pollen deposition by visiting bumblebees (*Bombus terrestris*) under lab conditions. We used three *Solanum* species with different floral morphologies and orientations: the tomato *Solanum lycopersicum* (Solanum section Lycopersicon (Mill.) Wettst.), *S. rostratum* Dunal (Solanum sect. Androceras Whalen) and *S. seaforthianum* Andrews section Dulcamara *sensu* (Nee, 1999); Dulcamaroid clade (Weese and Bohs, 2007; Gagnon et al., 2021). *S. lycopersicum* and *S. seaforthianum* are radially symmetrical with anthers surrounding the stigma and are both naturally pendant (Figure 5.1). Both species are self-compatible but *S. lycopersicum* benefits from bee pollination (Cooley and Vallejo-Marín, 2021). *S. rostratum* is zygomorphic, heterantherous, enantiostylous, horizontally oriented and dependent on outcrossing for reproduction (Vallejo-Marín et al., 2010; Vallejo-Marín et al., 2013). We ask the following questions: (i) Does orientation (Pendant vs Horizontal) affect total pollen deposition (self and outcross) in these three species? We expect that the manipulation of orientation will have a greater effect in the zygomorphic *S. rostratum* than in *S. lycopersicum* or *S. seaforthianum*, due to its requirement for more precise bee positioning on the flower. (ii) Does donor or recipient orientation affect outcross pollen deposition in *S. rostratum*? (iii) Does the correspondence between donor and recipient orientation affect outcross pollen deposition in *S. rostratum*. We predict that a mismatch between donor and recipient orientation will reduce outcross pollen deposition in *S. rostratum*.

5.3 Methods

5.3.1 Plant material

We studied three *Solanum* (Solanaceae) species with different floral orientations: *Solanum lycopersicum* L. (*Solanum* section *Lycopersicon* (Mill.) Wettst.), *S. rostratum* Dunal (Solanum sect. *Androceras* Whalen), and *S. seaforthianum* Andrews section Dulcamara *sensu* (Nee, 1999); Dulcamaroid clade (Weese and Bohs, 2007; Gagnon et al., 2021). These species were chosen for differences in floral morphology. Flowers of *S. lycopersicum* tomato are bright yellow, radially symmetrical, with a tightly fused anther cone around the central stigma (Glover et al., 2004) (Figure 1A). The natural orientation of *S. lycopersicum* flowers is pendant, i.e., downward-facing, with the flower's main axis perpendicular to the ground. For our experiments, we used the cherry tomato F1 hybrid "Sweet Million" (Premier Seeds Direct, Salisbury, UK). The flowers of this variety of cherry tomato are relatively small, with an average corolla width of approximately 2.2mm, and the stigma protruding just above the anther cone.

The second species, *S. seaforthianum* Andrews (section Dulcamara *sensu* (Nee, 1999); Dulcamaroid clade (Weese and Bohs, 2007; Gagnon et al., 2021)) is a vine native to tropical South America and cultivated or invasive in many parts of the world (Jagatheeswari, 2014). Flowers have relatively large, violet corollas, which open out more widely as flowers age (Figure 1B). Corollas have an average width of 23mm. They are radially symmetrical, with five yellow stamens, unfused but loosely grouped around an exerted style (average length: 10.1mm, and one stamen slightly longer than the other four (average lengths of 8.2mm and 6.7mm, respectively). They are naturally pendant in orientation, with the flower's main axis perpendicular to the ground. *S. seaforthianum* is self-compatible, but its mating system is unknown, and it is visited by bees, including *Eulaema* sp. (L.N. pers obs).

Finally, *S. rostratum* Dunal (*Solanum* sect. *Androceras* Whalen), is native to North America, with the highest genetic diversity found in Mexico, but also found in the southern U.S.A (Whalen, 1978; Zhao et al., 2013) and invasive in other parts of the world including China (Zhao et al., 2013). Flowers of *S. rostratum* are relatively large, yellow, zygomorphic, and heterantherous (they possess two types of anthers) (Todd, 1882; Bowers, 1975; Vallejo-Marín et al., 2010) (Figure 1C). These flowers have an average corolla width of approximately 28mm. One anther (pollinating anther) is usually darker in colour, longer, S-shaped, and the stamen has an average length of 14.3mm. This anther contributes proportionally more to pollination (Vallejo-Marin et al., 2009). The four remaining yellow anthers (feeding anthers) are shorter, straight, and each stamen has an average length of 9.7mm. and are more attractive to visiting bees while contributing proportionally less to pollination (Vallejo-Marin et al., 2009). Flowers are also enantiostylous: the style is deflected to either the left or the right in individual flowers, with the pollinating anther deflected to the opposite side (Jesson and Barrett, 2002). The natural orientation of these flowers is horizontal, with the main axis parallel to the ground (Whalen, 1978; Solís-Montero and Vallejo-Marín, 2017). *S. rostratum* is self-compatible, but predominantly outcrossing (Vallejo-Marín et al., 2013; Zhang et al., 2017). Wild populations are pollinated by a variety of medium and large-sized buzzing bee species, and flowers are also readily buzzed by captive bumblebees (Bowers, 1975; De Luca et al., 2013; Solís-Montero et al., 2015; Arroyo-Correa et al., 2019). Bees can position themselves in multiple ways to extract pollen, including buzzing one anther at a time, due to the flower's large size and loosely arranged stamens (Solís-Montero et al., 2015). Due to the spatial separation of anthers and stigma, bee size determines legitimacy as a pollinator: small bees (under 4mm) are considered pollen thieves as they do not generally contact the stigma during visitation (Solís-Montero et al., 2015; Solís-Montero and Vallejo-Marín, 2017). However, legitimate pollinators (larger than 4mm) contact both the pollinating anther and the stigma and are more constrained to specific positioning, i.e. grasping the feeding anthers with the body axis perpendicular to the main axis of the flower (Buchmann and Hurley, 1978; Solís-Montero et al., 2015; Solís-Montero and Vallejo-Marín, 2017).

Figure 5.1 Three buzz-pollinated *Solanum* species used in pollen deposition experiments: A: *S. lycopersicum* (pendant, Experiment One), B: *S. seaforthianum* (pendant, Experiment One), C: *S. rostratum* (horizontal, Experiments One and Two).

5.3.2 Plant growth

Plants were grown at the University of Stirling in the summer of 2020. Seeds of *S. rostratum* and *S. seaforthianum* were collected from wild populations in Mexico in 2010 and 2019, respectively. Seeds of *S. lycopersicum* were purchased from Premier Seeds Direct, Salisbury, UK. Seeds were treated with 1000ppm solution of gibberellic acid (GA3: Sigma-Aldrich, Dorset, UK) to induce germination, following Vallejo-Marin et al. (2014). Seeds were planted in modular seed compost (William Sinclair Horticulture PLC, Lincoln, UK) in seed trays, and kept in growth chambers for 4 weeks. Growth chambers were on a light-dark cycle of 18:6 hours, with a temperature of 28°C and 52% relative humidity. Seedlings were transplanted to 1.5L pots in a mix of All Purpose Growing Medium and Perlite (4:1 ratio, William Sinclair Horticulture PLC, Lincoln, UK). Plants were then kept in a pollinator-proof glasshouse, and fertilised weekly with Tomorite (Levington, Surrey, UK). Glasshouses were supplemented with artificial fluorescent lighting (16 hours of daylight), and supplemental heating was provided if temperature dropped below 14°C.

5.3.3 Bees

Experiments were performed using workers from two colonies of commercially obtained buff-tailed bumblebee (*Bombus terrestris audax*) (Biobest, supplied by Agralan Ltd, Swindon, UK). Colonies were provisioned with Biogluc sucrose solution underneath the colony (Biobest Group NV, Westerlo, Belgium) and ground honeybee-collected pollen (Agralan Ltd, Swindon, UK). Room temperature was 18-20°C.

5.3.4 Experimental set-up

Bumblebee colonies were attached to a grey-painted flight arena (60×60×37 cm) fitted with UV-transparent acrylic top and illuminated from above with an LED light panel (59.5×59.5 cm, 48W Daylight; Opus Lighting Technology, Birmingham, UK). Prior to experiments, bees gained experience freely foraging on *S. rostratum* flowers in the flight arena. Flowers were collected from the glasshouses in the morning of the experiments and presented in individual tubes filled with floral foam (OASIS Floral Products, Washington, UK). A nectar feeder containing 1M sucrose solution was also provided in the arena. In the experiments, we used two floral orientations for each species: Pendant and Horizontal. In the Pendant treatment, the main floral axis is perpendicular to the ground, while in the Horizontal treatment, it is parallel to the ground. Flowers of *S. lycopersicum* and *S. seaforthianum* are naturally pendant, while *S. rostratum* flowers are naturally horizontal. We used toothpicks and string to arrange flowers differently to their natural orientation. A naturally pendant flower stem was tied to a toothpick inside the floral foam tube to keep the flower horizontal. A naturally horizontal flower was tied to a toothpick which was positioned perpendicular to the tube. Flowers were positioned to be at a consistent height from the floor of the arena, regardless of orientation treatment.

5.3.5 Effect of floral orientation on total pollen deposition (self and outcross)

In the first experiment, we assessed the effect of floral orientation (Horizontal or Pendant) on total stigmatic pollen deposition, i.e., all flowers were intact, and pollen could be either self or outcross. We studied three plant species with differing natural orientations: *S. lycopersicum, S. rostratum* and *S. seaforthianum*. In each trial, bees were presented with between 15 – 35 flowers, depending on flower availability. Within a trial, all flowers were presented in the same orientation within the arena, either Horizontal or Pendant. Depending on availability, between 10 and 20 bees were released to freely forage on flowers for at least 60 minutes and up to a maximum of 90 minutes, unless foraging activity had clearly ceased by this time. After the trial had ended, styles were carefully removed from all flowers for stigma pollen counting.

5.3.6 Effect of complementarity of floral orientation on outcross pollen

In the second experiment, we assessed the effect of the correspondence between donor and recipient orientation on pollen transfer in *S. rostratum*. This species was chosen because it has a high outcrossing rate (70% outcrossing rate;(Vallejo-Marín et al., 2013)) and we were interested in the effect of orientation on outcross pollen deposition. Furthermore, the flowers are heterantherous and enantiostylous, and floral orientation is probably required for the correct alignment of pollinators necessary for mediating the function of the pollinating and feeding anthers (Vallejo-Marin et al., 2009), and cross-pollination (Jesson and Barrett, 2002). In our experiment, 75% of trials consisted of 12 flowers, in which 4 were pollen donors and 8 were pollen recipients. As flowers became more limited, later in the experiment, 25% of trials consisted of 9 flowers, in which 3 were pollen donors and 6 were recipients. We removed the styles from donors and glued shut the anthers of recipients with silicone glue. There were four combinations of donor-recipient orientations: Horizontal-Horizontal (H-H), Pendant-Horizontal (P-H), Horizontal-Pendant (H-P), and Pendant-Pendant (P-P). Each trial contained one of these combination treatments. Flowers were arranged randomly in the arena, and between 4-9 bees were released to freely forage for 30 minutes. After the trial had ended, styles were carefully removed from all flowers for stigma pollen counting.

5.3.7 Pollen counting

Stigmas were removed and mounted on microscope slides with melted fuchsin jelly for pollen staining (Kearns and Inouye, 1993). Total pollen loads on stigmas were counted under a binocular microscope (Olympus CX31, Tokyo) using 400x magnification.

5.3.8 Statistical analysis

We evaluated the effect of floral orientation on pollen loads in each stigma using generalised linear mixed models (GLMM) with a negative binomial error distribution and a log link function, using the *MASS* package. A negative binomial distribution was chosen to account for high levels of dispersion in the data (White and Bennetts, 1996). In all models, the response variable was pollen load in each stigma. For experiment one, a first model was fitted with an interaction term between floral orientation and plant species. The number of bees and flowers in each trial were used as covariates and trial number was used as a random effect. A second model was fitted without the interaction term. Akaike's Information Criterion (AIC in the *stats* package) was used to compare models. For experiment two, a first model was fitted with an interaction between donor orientation and recipient orientation. The numbers of bees, donors, and recipients in each trial were used as covariates and trial number was used as a random effect. A second model was fitted without the interaction term. AIC was used to compare models. The *DHARMa* package was used to produce residual diagnostic tests for each model (Hartig, 2019). Statistical significance of the main effects was assessed using the *car* package (Anova, Type II sums of squares) (Weisberg and Fox, 2011). All statistical analysis was performed in R 4.0.2 (R Development Core Team, 2021).

5.3.9 Ethics

Bumblebee experiments were approved by the Animal Welfare and Ethical Review Board at the University of Stirling.

5.4 Results

5.4.1 Floral orientation does not affect total pollen deposition (self and outcross)

In experiment one, in which all flowers were presented in the same orientation, we completed 19 trials using 423 flowers, of which 376 stigmas were collected for pollen counting (47 flowers were discarded due to falling on the floor of the arena during the trial, or stigmas being damaged). For *S. lycopersicum*, we collected 71 stigmas from 3 trials, 36 in Pendant treatment and 35 from the Horizontal treatment (Table 5.1). For *S. rostratum*, we collected 250 stigmas from 14 trials, 96 in Pendant treatment and 154 in Horizontal treatment (Table 5.1). For *S. seaforthianum*, we collected 55 stigmas from 2 trials, 35 in Pendant and 20 in Horizontal (Table 5.1). 42 stigmas were excluded from analysis due to issues with mounting or pollen visibility.

We found no interaction between floral orientation and plant species on pollen deposition (Table 5.2A). The interaction term was dropped from the model based on a comparison of models using Akaike's Information Criterion (AIC). In the simpler model, we found significant differences in pollen deposition among species (estimate: – 0.42, $p<0.01$) Table 2), with fewer pollen grains deposited on *S. rostratum* stigmas than *S. lycopersicum* or *S. seaforthianum* stigmas (Fig. 2, Table 2). (Figure 5.2 Table 5.2B). There was no effect of floral orientation on pollen deposition ($p<0.05$) (Figure 5.2, Table 5.2B).

	Horizontal			**Pendant**		
Species	**Mean ± s.e.**	**Range**	**n**	**Mean ± s.e.**	**Range**	**n**
S. lycopersicum	101 ± 8.3	5-250	35	108.5 ± 8.8	46-240	36
S. rostratum	59.7 ± 3.9	0-278	154	47.9 ± 3.8	5-212	96
S. seaforthianum	97.6 ± 24.7	0-180	20	134.5 ± 23.2	22-310	35

Table 5.1 Experiment one: total pollen deposition. Stigma pollen load means, standard errors, ranges, and sample sizes grouped by species.

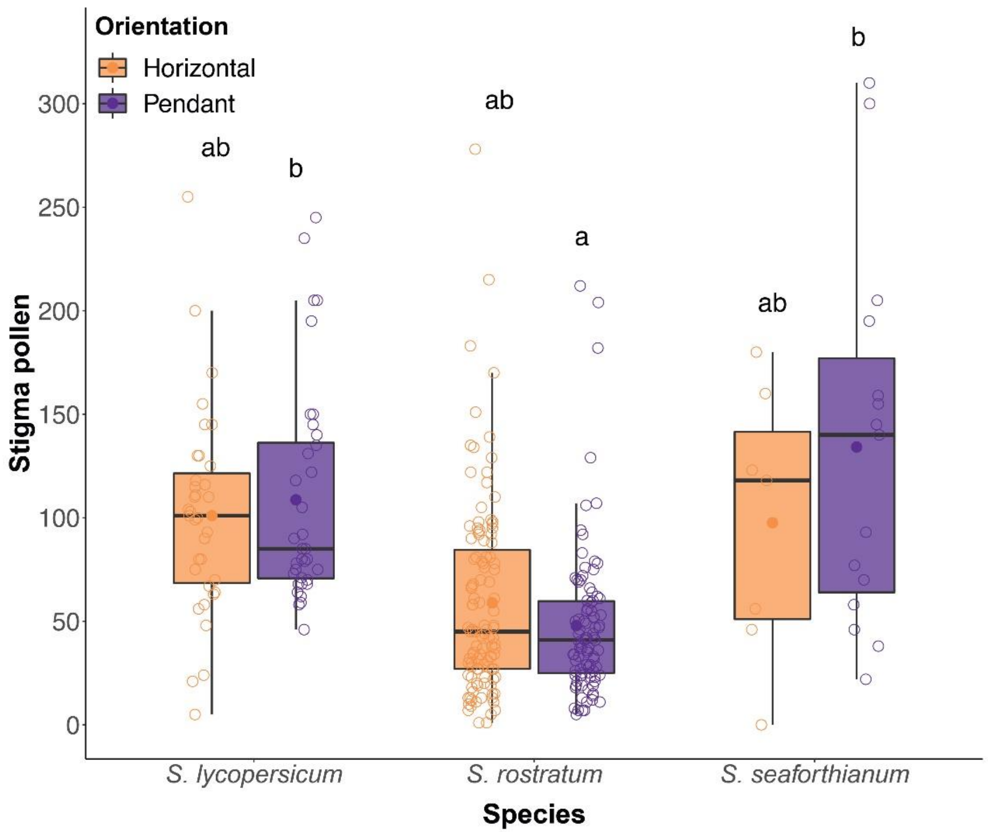


Figure 5.2 The effect of orientation on total (self and outcross) pollen deposition for three Solanum species: *S. lycopersicum*, *S. seaforthianum* and *S. rostratum*. Stigma pollen counts for each species are grouped by treatment (Horizontal or Pendant). *S. lycopersicum* (n = 35 in Horizontal, n = 36 in Pendant), *S. seaforthianum* (n = 20 in Horizontal, n = 35 in Pendant), *S. rostratum* (n=154 in Horizontal, n = 96 in Pendant).

Experiment one	**Estimate**	**Std. error**	***P*-value***	***P*-value****
A.				
Orientation:Species				0.59
Pendant:*S. rostratum*	-0.22	0.47	0.63	
Pendant:*S. seaforthianum*	0.2	0.68	0.77	
Orientation (Pendant)	0.13	0.43	0.77	0.91
Species				**<0.05**
S. rostratum	-0.42	0.37	0.27	
S. seaforthianum	-0.22	0.48	0.65	
No. flowers	0.02	0.02	0.33	0.33
No. bees	-0.03	0.02	0.2	0.2
B.				
Orientation (Pendant)	-0.02	0.15	0.91	0.91
Species				**<0.05**
S. rostratum	-0.55	0.21	**<0.01**	
S. seaforthianum	-0.09	0.3	0.76	
No. flowers	0.01	0.01	0.29	0.29
No. bees	-0.03	0.02	0.16	0.16

Table 5.2 Generalised linear mixed models (negative binomial error distribution) fitted for experiment one: total pollen deposition in three species. A: fitted with stigmatic pollen count as the response variable, an interaction term between orientation and species, orientation, species, number of flowers and number of bees as fixed effects, and trial number as a random effect. B: as A, without the interaction term. *P-value of fixed effect in linear model. **P-value calculated using Type II sums of squares. Sample sizes: *S. lycopersicum* (71), *S. rostratum* (241), *S. seaforthianum* (22).

5.4.2 Donor and recipient floral orientation affects outcross pollen deposition in S. rostratum

We completed 16 trials using 180 flowers (60 donors and 120 recipients), of which 119 recipient stigmas were collected for pollen counting (H-H: 30, H-P: 32, P-H: 29; P-P: 28) (Table 5.3). 6 stigmas were excluded from analysis due to issues with mounting or pollen visibility.

We found no interaction effect between donor and recipient orientation on outcross pollen deposition: the correspondence between donor and recipient orientation does not affect deposition (Table 5.4A, Figure 5.3 for all four trial combinations). The interaction term was dropped from the model based on Akaike's Information Criterion (AIC). In the simpler model, we found significant effects of donor (estimate: -0.47, p<0.05) and recipient (estimate: -0.69, p<0.01) orientations: flowers with either pendant donors or pendant recipients (or both) received less pollen (Table 5.4B, Figure 3).

Orientation	**Mean ± s.e.**	**Range**	**n**
Horizontal donor – Horizontal recipient	76.5 ± 15	4-280	30
Pendant donor – Horizontal recipient	58.9 ± 7.5	0-153	32
Horizontal donor – Pendant recipient	51.4 ± 13.3	1-249	29
Pendant donor – Pendant recipient	24.9 ± 5.5	1-144	28

Table 5.3 Experiment two: outcross pollen deposition. Stigma pollen load means, standard errors, ranges, and sample sizes for *S. rostratum*, grouped by orientation treatment.

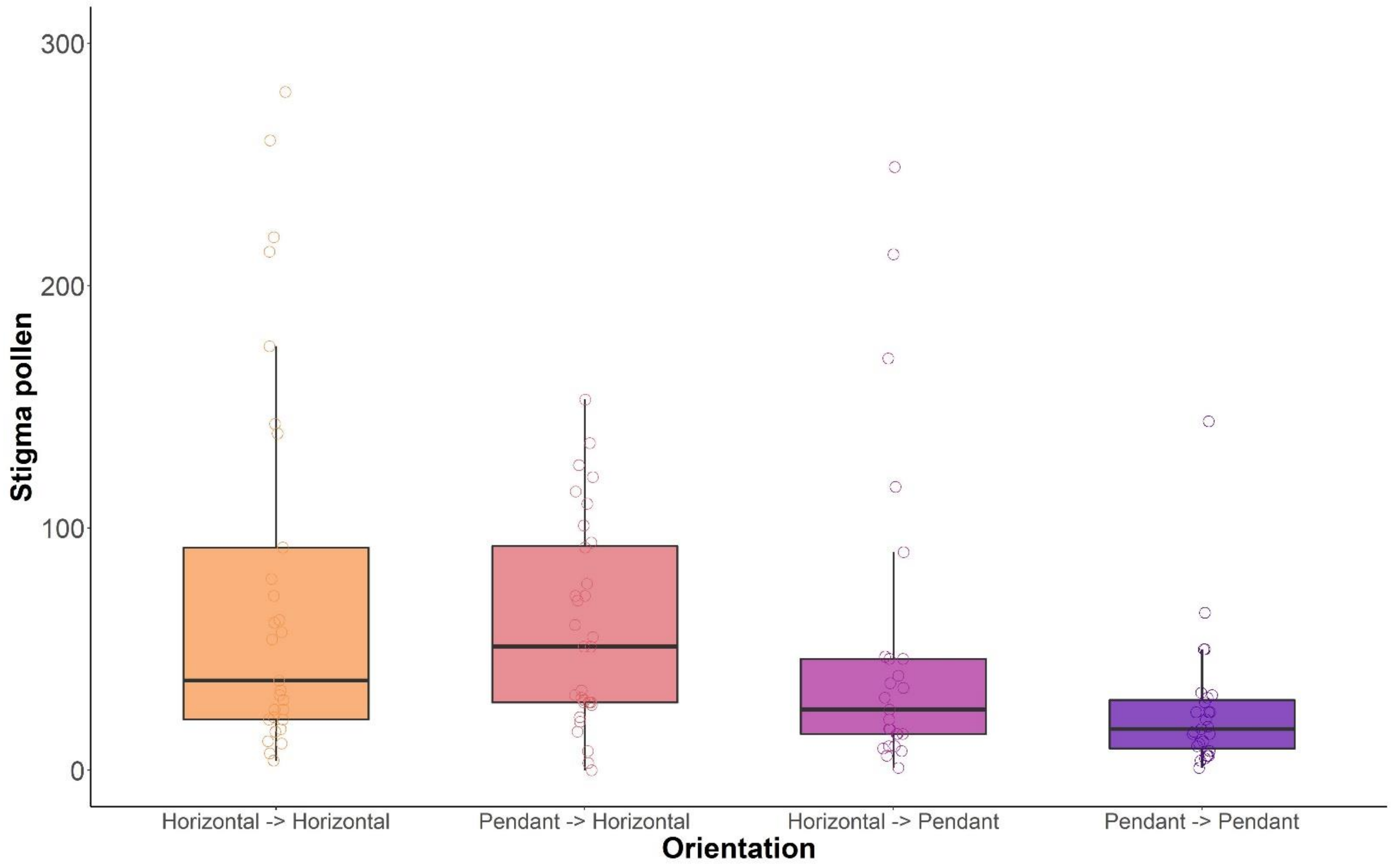
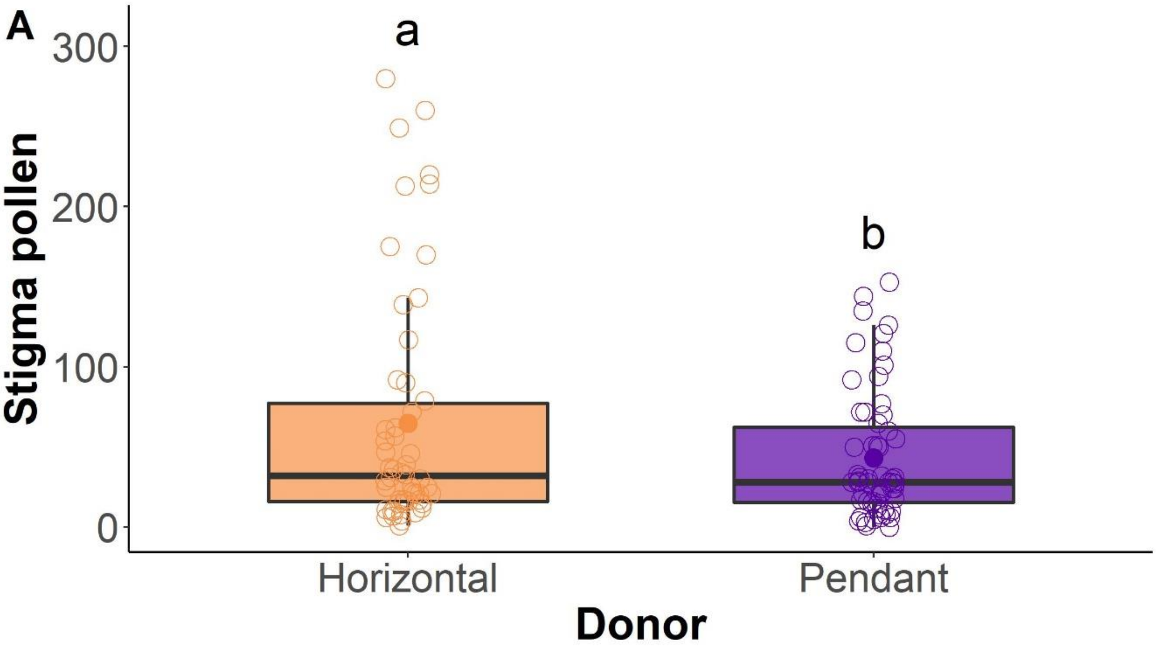


Figure 5.3 The effect of donor and recipient floral orientation on outcross pollen in *S. rostratum*. Stigma pollen counts for *S. rostratum*, grouped by orientation treatment (Horizontal-Horizontal (n = 29), Pendant-Horizontal (n = 25), Horizontal-Pendant (n = 32), Pendant-Pendant (n = 27)).



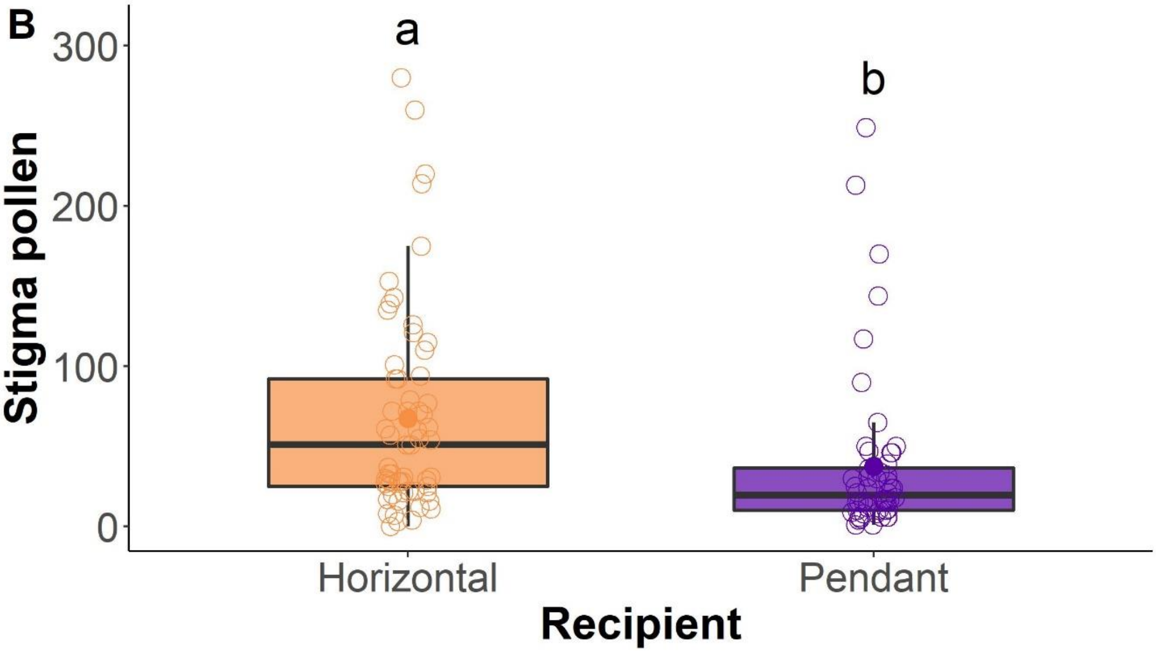


Figure 5.4 The effect of donor and recipient floral orientation on outcross pollen in *S. rostratum*. A. Stigma pollen counts when donor is either Horizontal (n = 54) or Pendant (n = 59). B. Stigma pollen counts when recipient is either Horizontal (61) or Pendant (52).

Experiment two	**Estimate**	**Std. error**	***P*-value**
A.			
Donor:Recipient	-0.72	0.42	0.09
Donor – Pendant	-0.08	0.3	0.78
Recipient - Pendant	0.34	0.29	0.23
No. donors	0.82	0.67	0.22
No. recipients	0.17	0.32	0.59
No. bees	0.14	0.07	0.06
B.			
Donor – Pendant	-0.47	0.23	**<0.05**
Recipient – Pendant	-0.69	0.22	**<0.01**
No. donors	0.82	0.76	0.28
No. recipients	-0.19	0.36	0.6
No. bees	-0.1	0.07	0.17

Table 5.4 Generalised linear mixed models (negative binomial error distribution) fitted for experiment two: outcross pollen deposition. A: fitted with stigmatic pollen count as the response variable, an interaction term between donor orientation and recipient orientation, number of donors, number of recipients and number of bees as fixed effects, and trial number as a random effect. B: as A, without the interaction term. P-values calculated using Type II sums of squares. Sample size: 113.

5.5 Discussion

Bilateral flowers are thought to require more precise pollinator positioning than radial flowers for effective pollen transfer and are often oriented horizontally to constrain the intrafloral position of visitors (Giurfa et al., 1999; Fenster et al., 2009). However, the effect of orientation on self and outcross pollen transfer in buzz-pollinated plant species has been seldom explored. Here, we investigate this by manipulating the floral orientation of three buzz-pollinated *Solanum* species with different floral symmetries and orientations and measuring subsequent pollen deposition by foraging bumblebees. We hypothesised that orientation impacts pollen transfer in heterantherous (bilateral) flowers via its effect on pollinator positioning. Our results from the outcrossing experiment suggest that floral orientation in heterantherous (bilateral) flowers can affect the deposition of outcross pollen. Recipient flowers received less pollen when they or the donor flowers were pendant, with the least amount of pollen received when both were pendant. The recipient orientation appears to have more effect than the donor orientation: pendant recipients with horizontal donors received substantially less pollen than vice versa. On the other hand, we found no effect of orientation on total pollen deposited (including self-pollen) in any of the species. We propose that the horizontal orientation of heterantherous flowers plays an important role in outcrossing, in addition to floral morphology, by ensuring effective pollen transfer, specifically stigmatic pollen deposition.

Our manipulation of floral orientation did not affect total (self and outcross) pollen deposition in any of the three species *S. lycopersicum S. rostratum*, *S. seaforthianum* (Figure 2.2). This is contrary to our expectation that *S. rostratum* flowers would experience reduced pollen deposition when oriented pendantly, because of the reduction in the chances of stigma contact between bee and flower. Previous work has shown that effective pollen deposition (including self and outcross) in the heterantherous, bilateral *S. rostratum* requires a precise fit between flower and pollinator (Solís-Montero and Vallejo-Marín, 2017), similar to other buzz-pollinated species (Mesquita-Neto et al., 2021). There are several plausible explanations for our result. First, all flowers in a single trial were oriented the same way: although bees could theoretically approach pendant flowers from multiple angles, they may have eventually adopted the same position on all flowers, enabling pollen to be released onto a suitable position on the bee's body for efficient transfer to the stigma of another flower. Second, a pendant orientation may provoke bees to move around on the flower, repeatedly changing position and increasing the chance of contact with the stigma. This may also increase the amount of time bees spend on pendant flowers compared to horizontal flowers. Self-pollen may therefore account for much of the pollen deposited, negating the outcrossing strategy of *S. rostratum*. The proportions of self and outcross pollen deposited might therefore differ between the two orientations. We are unable to confirm such explanations without further behavioural observations and separate quantification of self and outcross pollen, but these findings suggest that pollen release and deposition are not negatively affected overall by a pendant orientation, although the origin of the pollen being transferred is uncertain.

As expected, pollen deposition in the radially symmetrical flowers (*S. lycopersicum* and *S. seaforthianum*) was not affected by our manipulation of their orientation from pendant to horizontal (Figure 2.2). Interestingly, both radial, pendant species experienced higher average pollen deposition than *S. rostratum*, with a significant difference between *S. lycopersicum* and *S. rostratum*. It is possible that pollen transfer was more efficient across trials in these species, due to a reduced requirement for precise pollinator positioning in radial flowers (Neal et al., 1998). Pollen may also be released more easily in tomato flowers, in keeping with their ability to release some pollen when shaken by the wind (Hanna, 1999). As mentioned above for *S. rostratum*, much of the pollen deposited may be self-pollen and the proportions of outcross and self-pollen may also differ between species. We have not compared total pollen grain numbers across these plants, and we cannot rule out the possibility that tomato flowers of this variety contain more pollen than the other flowers.

In experiment two, we assessed the effect of orientation on outcross pollen deposition: recipient flowers were effectively emasculated (anthers glued shut), preventing self-pollination. A pendant orientation of either donor or recipient reduced outcross pollen deposition, with the lowest deposition when both were pendant (Figure 2. 3). This contrasts with experiment one, in which orientation had no effect and self-pollen may have been a significant contributor to pollen deposition. *S. rostratum* is mostly outcrossing (Vallejo-Marín et al., 2014), and its horizontal orientation is perhaps a strategy to promote outcross pollen transfer, although it does not prevent self-pollen transfer. In pendant flowers, the bees may have approached from multiple directions and positioned themselves in various ways to buzz, for example "upside-down", e.g., with the pollinating anther at the head of the bee. Additionally, bees may have spent less time trying to forage on the non-rewarding recipient flowers in this experiment, due to receiving no positive feedback, and may also have learned not to revisit. The chances of touching the stigma are therefore reduced. Although this is true for recipients of all orientations, horizontal recipients may still be more likely to receive pollen even in a short time frame, due to a more restricted initial pollinator position. The effect of pollen rewards on bee behaviour in this experiment cannot be confirmed here without further observations, for example trials in which there is a mixture of blocked and unblocked recipient flowers. There is evidence that bees spend less time on non-rewarding poricidal flowers (Buchmann and Cane, 1989), and perform fewer buzzes (Buchmann and Cane, 1989; Luo et al., 2008; Burkart et al., 2014) and shorter buzzes (Russell et al., 2016b), although Duncan et al found no differences in handling time in the field between flowers with blocked and unblocked anthers (Duncan et al., 2004). Bees can also learn to associate visual cues with pollen presence (Muth et al., 2016), which could extend to orientation, and are less likely to revisit non-rewarding flowers (Buchmann and Cane, 1989; Russell et al., 2015) although the relatively short time frame of our experimental trials may not be sufficient to elicit bumblebee learning of orientations.

We found that a pendant recipient orientation caused a greater reduction in pollen deposition than a pendant donor orientation (Figure 2.4), suggesting that the position of the stigma relative to the bee's body is under more constraints than the positions of the anthers, and highly reliant on a correct (horizontal) orientation for effective outcrossing. This fits with a recent study on the enantiostylous *Wachendorfia paniculate* (Haemodoraceae), showing that stigma position is "fine-tuned" to contact specific sites on the native pollinator's body (Minnaar and Anderson, 2021). This is also compatible with our above hypothesis: bees spent relatively less time on non-rewarding recipient flowers and foraged for extended periods on donors, perhaps negating an effect of donor orientation. This may have been exacerbated by the limited number of flowers available for foraging – bees likely revisited donor flowers multiple times until pollen was exhausted.

Contrary to expectation, we did not find a "mismatch" effect of orientation, which would be demonstrated by lower pollen deposition when the donor and recipient orientation were different. Instead, pollen deposition was not significantly different between horizontal/horizontal and pendant/horizontal and was lowest when both were pendant. We hypothesised that in mismatch trials, pollen would be deposited on an area of the body that is less likely to contact the recipient stigma (Solís-Montero and Vallejo-Marín, 2017; Minnaar and Anderson, 2021). As suggested above, bees may be changing position more than expected while foraging the donor flowers and spending more time on them per flower than on the non-rewarding recipient flowers.

Floral orientation in buzz-pollinated plants may have several drivers, including pollinator attraction (Makino and Thomson, 2012; Prokop et al., 2020) or pollinator partitioning (Wang et al., 2014a; Wang et al., 2014b). Within *Solanum*, as across angiosperms, a horizontal orientation is associated with bilateral symmetry and/or heteranthery (Bohs et al., 2007). Here, we provide evidence that orientation can affect pollen transfer in a naturally horizontal, bilaterally symmetrical *Solanum* species. Crucially, *Solanum rostratum* is reliant on outcrossing and has high outcrossing rates in the wild (Vallejo-Marín et al., 2013; Zhang et al., 2017). Its horizontal orientation likely promotes the transfer of outcross pollen relative to self-pollen (autogamy). We suggest that this is achieved through the interaction with the pollinator: the preferred position for bees (and the optimal position for pollen transfer) on heterantherous *Solanum* is upright, with the mandibles curled around the feeding anthers. Bees approaching horizontal flowers are already upright and can easily land in this position or move into it with little effort (Fenster et al., 2009; Naghiloo et al., 2020). Alternatively, a pendant orientation could undermine the outcrossing strategy. Bees approaching pendant flowers can approach from multiple directions and may buzz in a different position or take longer to change position, perhaps also transferring self-pollen in the process (Naghiloo et al., 2020). The relative proportions of outcross and self-pollen transferred in heterantherous *Solanum* species in the wild are unknown, but experiments on the horizontally oriented, heterantherous *Melastoma malabathricum* have shown low levels of bee-assisted self-pollination (Luo et al., 2008).

Following the same logic, orientation likely also affects the division-of-labour function of heteranthery, although we did not investigate this explicitly. Effective division-of-labour relies on pollen from the pollinating anther being transferred effectively, often via “safe sites” on the bee’s body which cannot be easily groomed (Vallejo-Marin et al., 2009; Koch et al., 2017; Tong and Huang, 2018). Our results suggest that either the relative proportions of pollinating and feeding pollen transferred or the total amount of pollen on the bee's body available after grooming, may change with a manipulation of floral orientation. Although in *S. rostratum* both anther types produce viable pollen (Vallejo-Marin et al., 2009), this could have negative fitness consequences for those plant taxa with reduced pollen viability in feeding anthers (Nepi et al., 2008; Luo et al., 2009). Similarly, we did not account for enantiostyly in these experiments (left and right morphs were picked at random for both donors and recipients), which may have been played a role in our results (Jesson and Barrett, 2002). A pendant orientation likely undermines the outcrossing function of enantiostyly by increasing the proportion of pollen transferred to flowers on the same plant (geitonogamy): pollen from the pollinating anther could land on either side of the bee's body and this pollen could be transferred to the stigma on the same morph. Furthermore, we did not consider the effect of a vertical orientation on pollination. Within *Solanum*, there are species such as the heterantherous *S. houstonii*, which display flowers almost vertically, potentially allowing bees to approach and land from multiple directions (Fenster et al., 2009; Naghiloo et al., 2020). Benefits of a vertical orientation may include increased attractiveness to pollinators (but may also increase visits from non-pollinators) (Makino and Thomson, 2012), or microclimatic effects, such as increased dehydration of anthers promoting pollen release (Corbet et al., 1988). *S. houstonii* is also andromonoecious: most flowers are male (staminate) and a few are hermaphrodites (male and female) (Anderson and Symon, 1989). This itself reduces the chances of autogamy and geitonogamy and increases outcrossing (Anderson and Symon, 1989; Papaj et al., 2017; Carbonell, 2019) and may intensify the effect of orientation on pollen transfer: hermaphrodite flowers may require more precise pollinator positioning than male (staminate) flowers.

This study suggests that floral orientation and symmetry interact to affect pollen transfer in buzz-pollinated plants. Radial flowers appear to enable effective pollen transfer, regardless of floral orientation. In bilaterally symmetrical flowers, orientation plays an important role and may be constrained by their requirement for outcrossing.

Chapter Six: General Discussion

Distinct floral morphologies have repeatedly evolved across buzz-pollinated lineages, including the widespread cone flower and flowers with marked stamen dimorphism (Faegri, 1986; Bohs et al., 2007), yet little is known about the function of these morphologies. The effects of these morphologies on plant-pollinator interactions must be considered in the context of the relationship between poricidal flowers and buzzing bees, which entails specific biomechanical and behavioural constraints. Cone-like flowers may promote consistency, both in their vibrational properties and their effect on bee behaviour, whilst non-cone flowers are less uniform in their interactions (Nevard et al., 2021; Vallejo-Marín et al., 2021). In heterantherous flowers, a lack of uniformity across interactions may be related to the division of labour between stamen types (Dellinger et al., 2019a; Brito et al., 2021). In their responses to contrasting morphologies, foraging bees can be flexible in their foraging decisions and intrafloral behaviour (Papaj et al., 2017), but are more constrained in their buzz characteristics and appear unable to exploit the biomechanical characteristics of poricidal flowers (Switzer et al., 2019).

Here, I investigated buzz-pollinated flowers with different morphologies in the context of their biomechanical properties and their effects on bee foraging behaviour. In Chapter Two, I measured the transmission of artificial vibrations in flowers with different stamen arrangements. To my knowledge, this is the first study to directly investigate vibration transmission in different buzz-pollinated floral morphologies. I expected that cone-like (tightly held) flowers would alter vibrations less during their transmission than flowers with looser stamen arrangements. Based on previous studies, I also expected that amplitude would vary more than frequency due to transmission (King and Buchmann, 1996; Arroyo-Correa et al., 2019). Both expectations were met. Peak frequency was conserved across all flower types, whilst non-cone flowers amplified vibrations in some of their anthers, specifically those furthest from the source of vibrations. Cone-like flowers instead transmit vibrations faithfully across all stamens. The anther-fused cone, *S. dulcamara*, transmitted vibrations slightly more consistently than the filament-fused cone, *C. persicum*, indicating that the type of stamen fusion has little effect. The disparity between *S. houstonii* and *Exacum affine* was not as large as expected, given the substantial difference in non-focal anther length. Variation in both species was high, although the non-focal anther heterantherous *S. houstonii* experienced the highest amplitudes, whilst in *Exacum affine* vibrations were occasionally *attenuated* during transmission. Regardless of stamen arrangement, vibrations performed on one anther were successfully transmitted to all anthers. This is especially relevant for those flowers, especially heterantherous types, in which bees are able to buzz anthers separately (Papaj et al., 2017; Solís-Montero and Vallejo-Marín, 2017).

In Chapter Three, I measured the natural frequency of stamens in six heterantherous *Solanum* taxa, using a method based on cantilever theory (King and Buchmann, 1996). Natural frequency comparisons have mostly been made in wind-pollinated systems, in which it is a key trait regulating the evolution of pollination mechanisms (Timerman and Barrett, 2019). Yet its significance in buzz pollination is somewhat understudied. As expected, I found that longer stamens, those in larger, outcrossing taxa, had lower natural frequencies than those in smaller, selfing taxa. Contrary to expectation, and unlike Nunes et al. (2021), I did not find differences in natural frequency between stamen types, regardless of the level of heteranthery and despite differences in length and stiffness, demonstrating the limitations of this method. The frequencies I calculated overlap with bee vibrations, but at the low end, particularly in the large-flowered, outcrossing taxa. This could be relevant for the pollen dispensing function of stamens in these flowers. Likewise, biomechanical differences between stamen types may contribute to the division of labour observed in heterantherous flowers (Luo et al., 2008; Vallejo-Marin et al., 2009).

In Chapter Four, I investigated the buzzing behaviour of bumblebees (*Bombus terrestris)* on two of the species used in Chapter Three: the large, highly heterantherous, outcrossing *S. citrullifolium* and the small, weakly heterantherous, selfing *S. heterodoxum*. My results from Chapter Three show that these species differ in natural frequency, without overlap: *S. citrullifolium* stamens have substantially lower natural frequencies than *S. heterodoxum* stamens. Theoretically, bees might increase their foraging efficiency by adjusting their buzz frequencies to match these different frequencies. Some studies have indicated that bees buzz at different frequencies on different plant species (Corbet and Huang, 2014; Switzer and Combes, 2017), whilst others have found no effect (King and Buchmann, 1996; Arroyo-Correa et al., 2019). On the other hand, bees are known to respond to pollen release, which varies between plant species, and may mediate the effect of experience on their buzzing behaviour (Russell et al., 2016b; Switzer et al., 2019). I found a slight difference in buzz frequency between species, but frequencies used on both species were far above their natural frequencies. Bees also performed fewer and shorter buzzes on *S. heterodoxum*. Handling experience had little effect on buzz characteristics. These small differences in buzzing behaviour may be related to differences in pollen quantity and release patterns between species (Kemp and Vallejo-Marín, 2021).

In Chapter Five, I investigated the effect of floral orientation on bee-mediated pollen deposition in flowers with bilateral and radial symmetry. Bilateral flowers require precise pollinator positioning, which is aided by a horizontal orientation (Neal et al., 1998; Armbruster and Muchhala, 2020). Within the context of buzz-pollinated morphologies, cone flowers are radially symmetric and often pendant, whilst heterantherous flowers are bilaterally symmetric and generally horizontal (Faegri, 1986; Bohs et al., 2007). Consequently, I expected that manipulating the orientation of bilateral, heterantherous flowers (*S. rostratum*, horizontal → pendant) would have a greater detrimental effect on *total* pollen deposition than in radial, cone-like flowers (*S. lycopersicum* and *S. seaforthianum*, pendant → horizontal). Contrary to this prediction, I found no effect of orientation on total pollen deposition in any of the species. I also predicted that a mismatch between donor and recipient orientation would have a negative effect on *outcross* pollen deposition in bilateral flowers. I found that the mismatch had no effect, but a pendant orientation in either donor or recipient, or both, reduced pollen deposition. Given that *S. rostratum* is predominantly outcrossing, this suggests an important role for horizontal orientation in promoting outcrossing in heterantherous, buzz-pollinated flowers (Vallejo-Marín et al., 2010).

This thesis has examined two, interrelated axes of variation in buzz-pollinated flowers, stamen arrangement and stamen dimorphism, and their effects on various aspects of the buzz-pollination interaction, including biomechanical properties and bee foraging behaviour. In the following sections, I will discuss a) the functional implications of different stamen arrangement types; b) the role of stamen dimorphism in plant-pollinator interactions; c) pollen foraging and bee responses to different floral morphologies.

6.1 The function of stamen arrangement in buzz pollination

The arrangement of stamens within the androecium of buzz pollinated flowers varies from a tight cone to highly separated, freely moving stamens, with many permutations in between (Glover et al., 2004; Bohs et al., 2007; Gavrutenko et al., 2020). I propose that *bee-dependent* (i.e., not primarily selfing) species can be categorised into three broad "types", each with distinct biomechanical properties and effects on bee behaviour: cone, loose, and heterantherous (Figure 6.1). Within my thesis, I have compared different species of the types: cone (*C. persicum, S. dulcamara, S. lycopersicum, S. seaforthianum*), loose (*E. affine*), stamen-dimorphic / heterantherous (*S. houstonii, S. rostratum, S. citrullifolium, S. grayi grandiflorum, S. fructo-tecto, S. heterodoxum, S. grayi grayi*).

The cone, or "cone-like", flower type, convergently evolved across lineages, is emblematic of the buzz pollination syndrome, and may represent an adaptive peak (Faegri, 1986; Harder and Barclay, 1994) (Figure 6.1A). The cone has isomorphic stamens, low herkogamy relative to the other types, is radially symmetric and often pendant (Faegri, 1986; Glover et al., 2004). We can distinguish the cone type as an arrangement in which the anthers must be buzzed as a whole, because they are fused, or are almost always buzzed as a whole (Glover et al., 2004). For instance, *S. seaforthianum* stamens are unfused but bees buzz the entire cone (pers.obs.), the style is central and anther pores are directed towards the centre, indicating that it functions as a cone flower. Glover (2004) argues that this type of cone may be susceptible to pollen wastage due to anther movement during bee manipulation, directing the pollen away from the bee's ventral side – however, empirical evidence for or against this hypothesis is lacking. I suggest that both cone and cone-like flowers benefit from consistency within and between interactions, arguably functioning as a single unit rather than individual stamens. External vibrations are faithfully conserved across the cone (Chapter Two), and this is little affected by fusion type (anthers fused or filaments fused). Similarly, Vallejo-Marin (2021, preprint) recently found that in flowers with experimentally glued anther cones, transmission was affected least in flowers which already had (unfused) cones. Interestingly, they also found that vibrations were amplified between focal and non-focal anthers in experimentally fused cones. This apparent conflict with my results could be due to morphological differences between the species used in each study. In any case, although bees commonly grasp the whole cone while buzzing, reducing the need for anther-anther transmission, the vibration point may still vary with the position of the bee.

The bee’s limited intrafloral behaviour is a key benefit of the cone flower: they are pendantly oriented, allowing bee approach/positioning from multiple directions, suggesting there is no need for precise pollinator positioning (Faegri, 1986; Neal et al., 1998). My results on pollen deposition (Chapter Five) lend support to this hypothesis, showing that pollen transfer in cone flowers remains consistent despite manipulation of their floral orientation. This is the case for *S. lycopersicum* (fused cone) and *S. seaforthianum* (anthers held together without fusion). Pollen from cones is ejected onto the bee’s ventral side, which then contacts the stigma, likely in every buzzing interaction (Figure 6.2A). The high chance of stigma contact may outweigh any downsides to this strategy, for instance a higher chance of heterospecific pollen deposition due to relative generalisation (Arceo-Gómez et al., 2016), or the grooming behaviour of bees, who may groom most of the pollen from their abdomens prior to visiting the next flower (Koch et al., 2017; Tong and Huang, 2018).However, we did not measure *outcross* pollen deposition specifically, as in *S. rostratum*, so it is unknown whether the proportions of self and outcross pollen change with orientation. It remains possible that a pendant orientation promotes self-pollen transfer, perhaps facilitated via bee behaviour on the flower, providing some reproductive assurance in self-compatible species. There may also be other advantages to a pendant orientation, for instance the exclusion of detrimental non-buzzing visitors unable to hang upside-down onto the flower (Wang et al., 2014a; Prokop et al., 2020). Vallejo-Marin (2021) found that experimentally fused cone flowers also release more pollen than “loose” flowers, indicating that stamen arrangement affects their dispensing function. The cone flower may be favoured in an environment with few pollinators, in which pollen dispensing between multiple visitors is not possible, but flowers can expect high pollen transfer by each individual (Harder and Wilson, 1994). Evolution away from this morphology may be driven by a need for greater specialisation on a subset of bees (Yoder et al., 2020) or an increased need for herkogamy to reduce the occurrence of selfing (Webb and Lloyd, 1986).

The distinction between “cone” and “loose” flower types is potentially fuzzy, but I would define loose flowers as those with isomorphic anthers and high herkogamy relative to cone flowers (Figure 6.1B). In these flowers, bees can, and commonly do, buzz only one or a few anthers at a time. An important caveat is that this definition requires a thorough knowledge of pollinator types and behaviour, which is missing for many buzz-pollinated species, although some general behavioural traits can be inferred. For instance, larger bees may be more likely than smaller bees to treat loose flowers as a cone, buzzing all anthers at once (pers.obs.). The relationship between floral morphology and plant-bee interactions is thus somewhat contingent on the pollinator environment. Bee behaviour on non-cone flowers is also highly flexible within individuals (Papaj et al., 2017), and this variation may be an important feature of pollination in loose flowers. This type is less studied than the others, perhaps because it is trickier to delimit, and I only considered one example of it in this thesis: *Exacum affine*. In this species, vibrations change during transmission, unlike in cone flowers. The non-focal anther experiences amplified vibrations, although there is considerable variation in this effect, and vibrations were attenuated in some instances. A bee buzzing a single anther may thus cause pollen wastage from a different anther, or this self-pollen may fall onto the stigma, which is situated below the anther pores. The behaviour of wild pollinators of this species is unknown, but bees of around the size of *Bombus* spp. are able to buzz its anthers all at once (Russell et al., 2016a). Interestingly, *E. affine* has weak bilateral symmetry, due to the style's position and the curvature of the anthers. It is also horizontally oriented, indicating a requirement for precise pollinator positioning (Fenster et al., 2009), although I did not include it in my pollen deposition experiments. Bees approaching this flower head-on are likely to contact the stigma en route to buzzing the anthers.

One potential function of looser anthers could be to aid pollen dispensing by reducing the amount of pollen released at once: Vallejo-Marin (2021, preprint) found that less pollen was released from loose flowers than when they were experimentally fused into a cone. Furthermore, buzzing a single anther rather than all the anthers theoretically limits the amount of pollen removed per unit time. This dispensing strategy may be particularly relevant in flowers with extremely separated stamens, i.e. those which cannot be buzzed efficiently as a group, as seen in many melastomes (Melo et al., 2021). This type of loose flower was not considered here but may show differences in its effect on bee foraging behaviour. For example, bees obliged to buzz stamens separately may spend more time on the flower to collect pollen, meanwhile changing position and increasing the chances of stigma contact.

The third flower type which I considered in this thesis is the heterantherous type. These flowers have dimorphic stamens, high herkogamy, bilateral symmetry, are generally horizontally oriented and often large relative to cone flowers (Bohs et al., 2007; Dellinger et al., 2019a)(Figure 6.1C and D). Crucially, although stamens are dimorphic, they can be held relatively tightly together, as in *S. houstonii*, and each type is commonly buzzed as a unit, at least by legitimate pollinators (pers.obs.) (Solís-Montero and Vallejo-Marín, 2017). Heterantherous flowers are set up for the feeding anthers to be buzzed while the pollinating anther releases pollen onto bee safe sites (Vallejo-Marin et al., 2009; Papaj et al., 2017) (Figure 6.2B). In *S. houstonii,* the pollinating anthers experience high vibration amplitudes when a feeding anther is vibrated (Chapter Two). This transmission (and amplification) of vibrations through the tightly arranged androecium helps to ensure that pollen is released from the pollinating anthers when bees are foraging from feeding anthers. Although the three pollinating stamens of *S.houstonii* appear to be a single unit, the tips of their anthers oscillate somewhat separately when the flower is vibrated, in contrast to species like *S. rostratum* and *S. citrullifolium* with only pollinating anther (pers.obs.). This may help to ensure the release of pollen over a sufficiently wide area on the bee's dorsal side, especially given the narrowness of each anther alone, although it may also increase pollen wastage.

Legitimate pollinators of heterantherous flowers need to be large enough to contact both the pollinating anther(s) and the stigma (Mesquita-Neto et al., 2017; Solís-Montero and Vallejo-Marín, 2017). Furthermore, these flowers are bilaterally symmetrical and likely require precise pollinator positioning (Neal et al., 1998; Bohs et al., 2007). Unlike radial cone flowers, heterantherous flowers therefore tend to be horizontally oriented to limit the bee's initial approach and position (Fenster et al., 2009). My pollen deposition results lend support to the role of orientation in pollinator positioning: specifically, bees transfer less outcrossed pollen when naturally horizontal *S. rostratum* flowers are pendant. It is unclear whether inaccurate pollinator positioning also increases the transfer of self-pollen through differences in intrafloral behaviour. Due to size and position requirements for legitimacy of pollinators, many visitors to heterantherous flowers, even by buzzing bees, are illegitimate or inefficient (Solís-Montero et al., 2015).

The transmission of vibrations in these species can affect the severity of these visits. For instance, small bees buzzing feeding anthers without contacting the pollinating anthers will cause pollinating pollen to be released and potentially wasted. The pollinating anthers of *S. houstonii* may counteract this by spreading pollen in a relatively wide area, turning even a small bee into a legitimate pollen vector, assuming they then contact the stigma of another flower, perhaps whilst buzzing the pollinating anthers (Figure 6.2C). Specifically, *S. houstonii* flowers are often oriented almost-vertically, impacting pollinator positioning by allowing bees to approach from multiple directions and perhaps increasing the chance of stigma contact.

One limitation of my thesis is that my examples of heterantherous (and loose) flowers were still relatively tightly arranged, relative to the whole range of buzz-pollinated floral morphologies. Heterantherous flowers with highly separated stamens are common in the Melastomataceae (Melo et al., 2021). My results suggest two ways in which such flowers could differ from more tightly arranged heterantherous flowers in their effects on plant-pollinator interactions. First, they may not transmit vibrations in the same way – the physical separation of several stamens within a large corolla may dampen vibrations from one anther (i.e. the one being buzzed) to another. This may play a role in the evolution of stamen appendages, such as those seen in *Huberia bradeana*, which appear to aid vibration transmission and pollination (Bochorny et al., 2021). Second, the orientation of these flowers may be less constrained by a requirement for precise pollinator positioning, due to the widespread arrangement of pollinating stamens and the assumption that bees are less able or likely to buzz all feeding stamens together. This hypothesis is in line with Konzmann et al (2020), who propose that in many melastome species (often with loosely arranged stamens) heteranthery may function to accommodate bee visitors: differently sized stamens will contact bees of different sizes, increasing dispensing to multiple individuals and promoting pollen transfer.

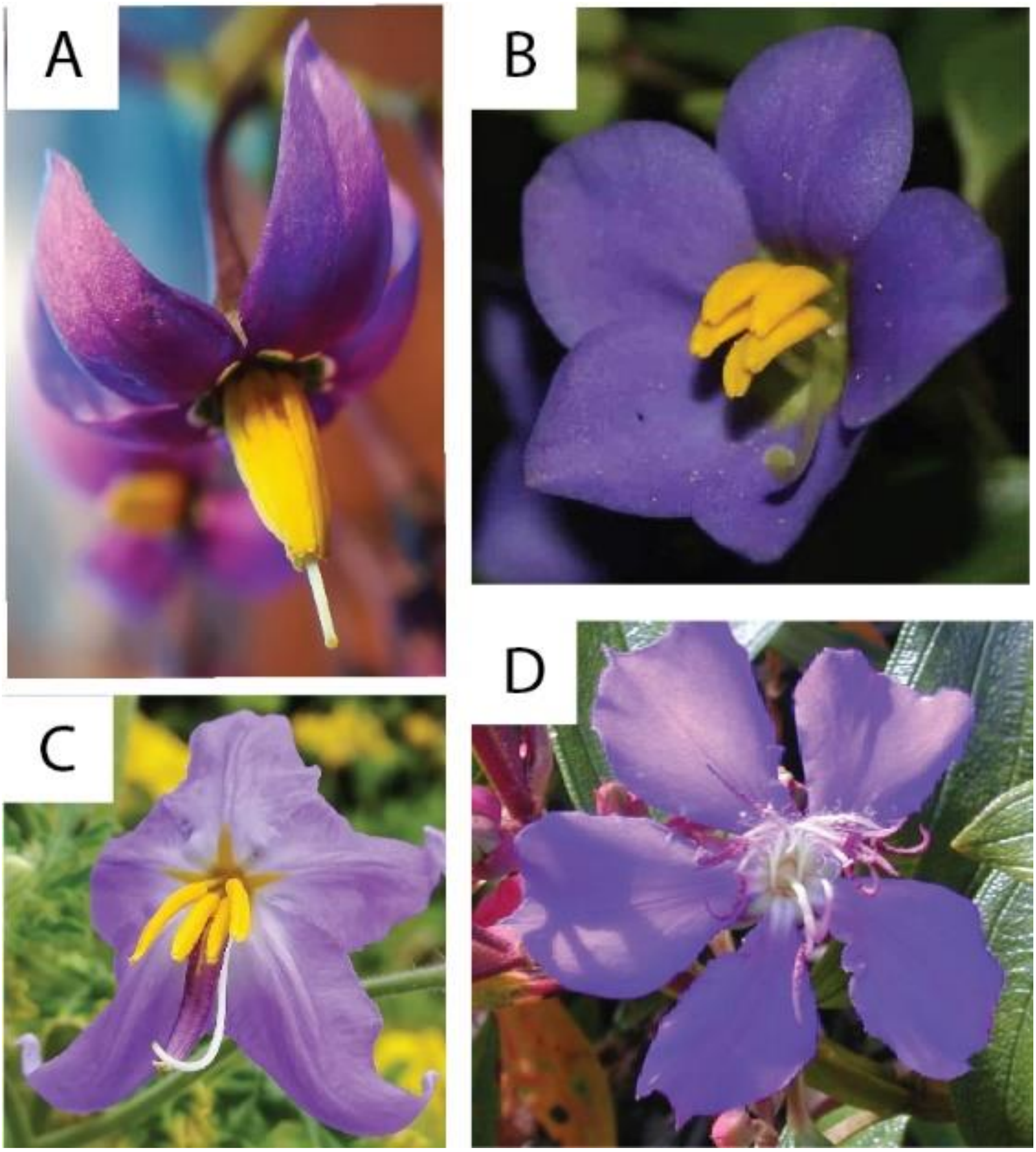


Figure 6.1 Three types of stamen arrangement in buzz-pollinated flowers. A: cone (*S. dulcamara)*; B: loose (*Exacum affine*); C: heterantherous (*S. citrullifolium*); D: heterantherous (*Pleroma granulosum*). C and D demonstrate differences in the spatial separation of stamens in heterantherous flowers.

6.2 Division of labour in heterantherous flowers

Stamen dimorphism, or heteranthery, is widely associated with a division of labour between pollinating anthers and feeding anthers in bee-pollinated plants. Alternative functions for heteranthery have been proposed (Dellinger et al., 2019a): for example, two sets of stamens may promote a gradual pollen presentation strategy when pollen availability in each set is temporally staggered (Kay et al., 2020). Yet across taxa, morphological differences between stamen types are often accompanied by functional differences, such as in pollen quantity (Luo et al., 2009; Pinheiro-Costa et al., 2018), viability (Nepi et al., 2008; Luo et al., 2009), or release rates (Brito et al., 2021), supporting the division of labour hypothesis in these systems.

Although many studies have investigated these differences, few have investigated differences in biomechanical properties between stamen types, despite the importance of biomechanics in buzz pollution interactions. Here, I found that pollinating stamens were more rigid than feeding stamens across all the species, including those with relatively weak heteranthery (Chapter Three). This is unsurprising, given the differences in morphology, size, and filament diameters between the stamens. Differences in rigidity may impact the response to vibrations between each stamen type: stiffer structures transmit vibrations more faithfully (Velilla et al., 2020). As discussed in the previous section, this may help promote pollen release from pollinating anthers when feeding anthers are being buzzed. It could also affect bee behaviour, for buzzing a single, flexible stamen may be a less stable position than buzzing the whole group (or indeed, buzzing the rigid pollinating anther). I did not find a difference in first natural frequency between stamen types, likely due to limitations of the cantilever model for measurement of curved structures (Chapter Three). Nunes et al. (2021) show in the same taxa that pollinating stamens have lower natural frequencies than feeding stamens. Whether this difference functions in the division of labour or is simply a side-effect of size differentiation, remains unexplored.

One way in which biomechanics may contribute to the division of labour is if biomechanical properties are linked to differences in pollen dispensing strategies between stamen types (Harder and Barclay, 1994; Dellinger et al., 2019a). The lower natural frequencies in pollinating stamens are further from bee vibrations than the natural frequencies of feeding stamens – this may help to limit pollen release in pollinating stamens, which rely more on dosing between individuals (Nunes et al., 2021). On the other hand, both may be sufficiently low, relative to bee vibrations, not to have any impact during bee foraging. The apparent lack of frequency-matching by bees may be beneficial for the dispensing strategy of these plants. There is some variation between bee taxa in their buzz frequencies (Burkart et al., 2011; De Luca et al., 2019) – some floral vibrations may inadvertently coincide with natural frequency of the feeding or pollinating stamens, even if individual bees do not adjust their vibration frequency. Moreover, the relationship between variation in pollen dispensing strategies and the division of labour is not universal amongst heterantherous species (Dellinger et al., 2019a; Telles et al., 2020; Brito et al., 2021) and investment in each stamen may depend on pollination context. The few comparisons of pollen release dynamics between stamen types have found conflicting patterns. Two studies show that pollinating stamens (in four species investigated) release pollen more quickly, arguing that this maximises pollen transfer by the first visitors whilst promoting further visits from bees foraging on feeding stamens which are gradually releasing pollen (Dellinger et al., 2019a; Brito et al., 2021). However, Telles et al. (2020) found the reverse: in their system, pollinating stamens release pollen more slowly, perhaps maximising the number of visitors to whom pollen is dispensed: even if feeding stamens are empty, bees will still visit as pollen availability is cryptic (Lunau, 2005). Such disparities in the division of labour may be linked to variation in pollinator visitation rates between species, however the empirical data to test this is lacking. Alternatively, they may reflect differences in anther structure, perhaps due to developmental constraints in different buzz-pollinated lineages.

This wide variation in the functional consequences of heteranthery raises questions regarding its linkage to morphological and biomechanical differences between species. How do differences between pollinating stamens (multiple vs one, or flexible vs rigid) impact heteranthery? Why are the pollinating anthers in species like *citrullifolium* or *rostratum* curved in such a particular way and how does it impact their response to vibrations and pollen dosing? Gathering empirical data to cover this biomechanical variation is naturally time-consuming. A promising alternative is the recent use of finite element modelling to assess different aspects of stamen biomechanics, such as natural frequencies (Jankauski et al., 2021, preprint). Model parameters can be altered to reflect differences in morphologies seen in buzz-pollinated species, although the link between these biomechanical properties and pollen dispensing will require additional modelling of pollen dynamics (Jankauski et al., 2021, preprint). An important advantage of such modelling approaches is the inclusion of bees in the interaction, which is harder to achieve during experimental assessment of biomechanical properties.

Studying the division of labour function in different floral morphologies will also be facilitated by advances in pollen labelling technology, such as the recent application of quantum dots. Quantum dots are semiconductor nanoparticles which can be attached to pollen grains, and emit light when exposed to UV (Minnaar and Anderson, 2019). They have been used to assess pollen transfer by bees visiting non-poricidal plants in a field setting (Konzmann et al., 2020) and outcrossing between flowers of different morphs (Minnaar and Anderson, 2021). Multiple quantum dot colours have been developed, with the potential for use to track the fates of pollen from pollinating and feeding anthers, shedding light on the division of labour. However, tagging pollen grains within poricidal anthers is trickier than in flowers with exposed pollen (pers. obs.), and the attachment of quantum dots to pollen may also be affected by the lack of pollenkitt in many buzz-pollinated species (Minnaar and Anderson, 2019).

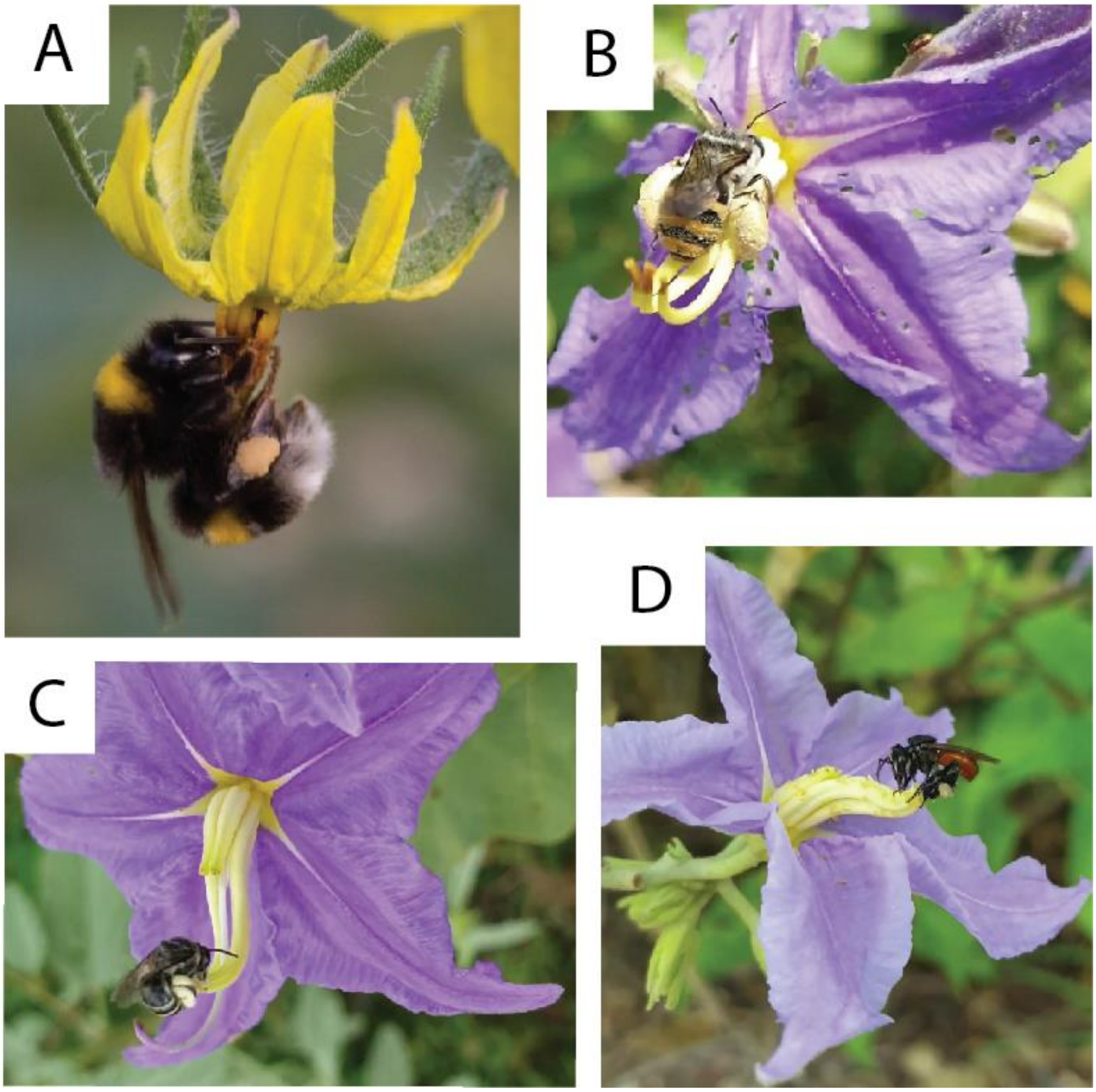


Figure 6.2 Bees demonstrating different foraging behaviours on contrasting flower types. A: *B. terrestris* buzzing the cone-shaped *S. lycopersicum*, all anthers at once. B: Colletid bee (likely to be a legitimate pollinator) buzzing the feeding anthers of the heterantherous *S. houstonii*, with the pollinating anthers angled towards her dorsal side. C: small colletid bee (likely an illegitimate visitor) buzzing the pollinating anthers of *S. houstonii*. D: *Trigona fulviventris* (a pollen thief) chewing the anthers of *S. houstonii* to collect pollen.

6.3 Floral morphology and bee foraging behaviour

Pollen foraging requires bees to use complex motor skills, and they benefit from learning complex floral morphologies to improve their foraging efficiency (Laverty, 1980; Raine and Chittka, 2007). Floral vibrations are particularly costly to produce, implying that bees visiting poricidal flowers are strongly incentivised to improve their efficiency (Russell et al., 2017). This may affect the decision to vibrate on specific poricidal flowers, in ways that are different to non-poricidal flowers, which provide pollen at less collection expense. My results from Chapter Four show that naïve bumblebees are reluctant to visit the small-flowered *S. heterodoxum,* likely due to visual or chemical cues. Importantly, most experienced foragers stopped visiting *S. heterodoxum* after two or three trials, perhaps because the flower was not rewarding enough to justify their foraging efforts, despite no alternative flowers being available. It is unclear whether this is due to the total amount of pollen available being low, or some other aspect, e.g. pollen characteristics (Nicholls and Hempel de Ibarra, 2017). It remains unexplored whether bees would make the same decision if the flower was not poricidal, i.e. pollen could be collected by scrabbling.

Bumblebees apparently do not exploit differences in stamen natural frequency between flower types: stamens in large flowers have lower natural frequencies than in smaller flowers (Chapter Four), but bees buzz at much higher frequencies, with only a small difference between flower types. Although I did not measure bee buzzing on specific stamen types, it seems implausible given my data and previous studies, that bees adjust their frequency between stamen types on the same flower (Switzer et al., 2019). Frequency-matching may not be a strategy employed by bees of this size, who may already be producing the vibrations which are most efficient for pollen collection (De Luca et al., 2013). For instance, buzzing at the maximum amplitude may be more efficient than buzzing at the natural frequency. I did not measure amplitude here, but it is also possible that bees demonstrated flexibility in their buzz amplitude between species or with handling experience (Arroyo-Correa et al., 2019). More broadly, there may be costs associated with the ability to change buzz characteristics, which constrain the evolution of behavioural flexibility. I did find a slight increase in buzz frequency in the smaller-flowered species and suggest that it may be due to negative feedback from flowers which are providing fast-diminishing pollen returns (Russell et al., 2016b; Switzer et al., 2019; Kemp and Vallejo-

Marín, 2021). Bees may also be increasing their amplitude, as found in previous studies (Russell et al., 2016b; Switzer et al., 2019). Any potential increase in energy costs, for a relatively small reward, may also contribute to the drop-off in visitation rates in this species.

Bee foraging behaviour is affected by floral orientation, as their approach direction on the flower is more constrained by a horizontal orientation (Fenster et al., 2009; Naghiloo et al., 2020). My results from Chapter Five show that floral orientation affects pollen deposition, possibly due to foragers adopting different positions on pendant flowers compared with horizontal flowers. It is unknown whether differences in position affect foraging efficiency from the bee’s perspective, but they but may affect the stability of the bee and potentially her ability to groom between vibrations (Harder and Barclay, 1994). Orientation could also affect the bee’s decision of which anthers to buzz, due to variability in approach direction or intrafloral stability. Further experiments should assess the relative efficiency of different intrafloral positions, and the factors involved in this choice. In cone flowers, this might be moot and not affect foraging efficiency, but in flowers with loose or heterantherous stamen arrangements, the choice of anthers may impact pollen collection.

6.4 Concluding remarks

Floral morphologies mediate complex interactions between buzz-pollinated plants and buzzing bees. Flowers which transmit vibrations faithfully, like cone flowers, may benefit from consistency between interactions. Bees foraging on these flowers are constrained to few positions but foraging on these flowers may be more efficient than on flowers with spatially separated stamens. Flowers which alter vibrations during transmission, whether amplified or attenuated, may find their pollen dispensing strategies affected. This could be particularly detrimental in heterantherous flowers, in which release rates can differ between stamen types. Future work should examine the transmission of vibrations across more diverse morphologies, including heterantherous flowers with loosely-held stamens, as well as differences in pollination strategies amongst these species. More broadly, research into the development of stamen architectures will help to explain the prevalence of different types across lineages. For instance, are there constraints to the evolution of the cone-flower in some lineages, or the evolution of heteranthery in other lineages? Future work should also investigate further the factors involved in bee foraging decisions. Why do they decide to buzz one single anther, or anthers all together? Why do they give up re-visiting flowers despite receiving pollen rewards? Crucially how do these decisions affect both foraging efficiency and pollination efficiency, and how might this balance change with variation in floral morphology?

Flower and bee have different interests regarding the fate of pollen: bees should collect as much as possible, while plants need to maximise the amount of pollen transferred. Floral morphology arguably alters the nature of this arrangement between flower and bee, on a spectrum from more mutualistic arrangements to more manipulative and exploitative conflicts. The cone flower may represent a relatively mutually beneficial relationship in which the interests of flower and bee converge, and both benefit from the consistency of pollen release in response to vibrations. Although bees can collect a large amount of pollen, all of which is made relatively available for feeding, the shape of the flower simultaneously maximises the chances of pollination by any pollen grains which inadvertently escape grooming. At the other end of the spectrum, heterantherous flowers are in a constant cycle of manipulation and exploitation. The horizontal orientation of these flowers manipulates bees to adopt a position optimal for the plant, and the difference in salience between stamen types further manipulates bees to buzz the feeding anthers. High flexibility in bee behaviour allows them to evade these attempts somewhat and act in their own interests, for instance by buzzing the pollinating anthers. However, the relative lack of flexibility in their buzz characteristics may limit the extent to which they can exploit the properties of different stamens. This allows poricidal flowers to employ pollen dispensing strategies in their own interest, with the caveat that they must continue to reward bees, who are fast learners quickly become disenchanted with unrewarding flowers. Differences in the balance of interests between flower and bee may be fundamental to the floral diversity we see in buzz-pollinated plants.

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Appendix A: Ethics form for Chapter Four

Animal Welfare and Ethical Review Body (AWERB)

Research Proposal Ethical Approval Form

SECTION A: Applicant details

A1. Name of applicant (principal researcher/student): Lucy Nevard
A2. Email address: lucy.nevard1@stir.ac.uk
A3. Faculty affiliation: Natural Sciences **Division/Research group: BES**
A4. Designation: Research Postgraduate
A5. STUDENTS ONLY **Course Name:** PhD **Academic Supervisor responsible for procedures:** Dr. Mario Vallejo-Marín
A6. RESEARCH STAFF ONLY **Job title:** Click here to enter job title
A7. Type of review required **ASPA ☐** **Non-ASPA ☒**
A8. Has a health and safety risk assessment been successfully completed **Yes ☒** **No ☐**

FOR ADMINISTRATIVE USE ONLY:

Ethics application reference numbers: AWERB/1718/162/New Non ASPA

Application complete, signed and dated ☒

AWERB decision **Date 09/08/2018**

Approved ☒ **Approved subject to minor amendments ☐**

Rejected ☐ **Deferred ☐**

Reason for rejection/details of required amendments: Click here to enter text

SECTION B: Project/protocol details

B1. Project/protocol title: Does bumblebee (*Bombus terrestris*) buzzing behaviour change with plant species?

B2. Project/protocol funding source: NERC Iapetus DTP
If 'other' please specify: Click here to enter text

B3. Proposed project/protocol start date: 12/07/2018

End date: 29/10/2018

B4. Short project/protocol description including the maximum length of the project:

Captive native bumblebee colonies (Bombus terrestris audax) will be sourced from Biobest (a commercial company providing bumblebees for pollination) and kept in the lab. These bees will be exposed to greenhouse-grown flowers in a large flight arena and buzzes recorded using a microphone and video camera. Individual bees used for the experiment will be captured temporarily and tagged with plastic numbered tags. At the end of the experiment, all bees will be euthanised by freezing at -80˚C.

The project will last up to four months.

B5. Provide a brief justification for the proposed study:

Buzz pollination (insect vibration of the flower to release pollen) is required for over 22,000 plant species. However, it remains unclear whether bees can adjust these vibrations to accommodate different floral morphologies. These experiments will address this question, and shed light on bumblebee cognition, floral evolution and pollination ecology.

B6. What are the study's main objectives?

To record bumblebee buzzes on different plant species and assess any differences in frequency, duration and amplitude.

B7. Provide details of the experimental design, the number of animals to be used and the statistical power of the proposed study, demonstrating that the benefits of the experiment outweigh the costs to the animal(s) involved:

3-5 colonies (approx. 60 workers from each) will be used. Multiple colonies are necessary to help account for colony level effects on individual behaviour. They will be kept in a lab at 20-22˚C with natural light from the windows.

Colonies will be fed ad libitum with sucrose solution (BioGluc 50% sucrose solution provided in a container underneath the colony) and the availability of this will be checked every few days by taking the colony out of the box. If Biogluc has run out (e.g. with an exceptionally large colony), it will be replaced. Colonies will also be fed regularly (every two-three days) with one tablespoon of ground commercial pollen prior to the experiment. Once the experiment starts, colonies will not be fed pollen, for a maximum of a week at a time, to motivate bees to forage for pollen. If necessary (to motivate the bees to leave the colony) the BioGluc will also be closed during the experiment and 1M sugar solution provided in the flight arena to feed the bees. Colonies will also be monitored for any unusual behaviour (e.g. extreme lethargy which may indicate starvation) or signs of disease. If the student (Lucy Nevard) is unable to feed the bees (e.g. due to illness), the supervisor (Mario Vallejo-Marin) or another student will be informed.

In the first stage of the experiment, bees will be trained to forage for pollen (from an artificial feeder) in the flight arena. This flight arena will be lit from above by an LED during the experiment. Trained bees will be caught in a ventilated tube with mesh at one end, and tagged on the thorax with plastic numbered tags using super glue. The bee will remain in the tube for up to five minutes, in order for the glue to dry. These tags do not affect the movement or flight of the bee. In the second stage, they will be exposed to flowers of one species of *Solanum*. Finally, they will be exposed to a different species of *Solanum* and individual buzzes on flowers will be recorded.

For each colony, data collection will end when it starts producing male bees (i.e. after about 6 weeks). Within three days of ending data collection, the colony will be euthanised by freezing at -80˚C.

The benefits of the experiments outweigh the costs to the animals, as the data will shed light on pollinator behaviour, which could have implications for plant/bee conservation and agriculture.

B8. How have the 3Rs been addressed?

Replacement: The experiment requires studying the vibrational behaviour of bees, and unfortunately there is no replacement to studying live bees.

Reduction: Individual bees will be recorded multiple times to gain the maximum amount of data points while minimising the number of bees required. The number of colonies to be used represents the trade-off between replication needed for statistical accuracy and utilising the fewest animals possible.

Refinement: The protocols follow on from previous experiments in this lab (over several years) and minimise harm to the bees. For instance, handling the bees in a way which minimises stress; tagging the bees in a non-harmful way; euthanising the bees as soon as possible after the experiment has ended.

B9a. If animals subject to ASPA regulations are to be killed, please confirm that this will be by a Schedule 1 method and name the appropriately trained person who will perform this:

NA

B9b. If animals not subject to ASPA regulations are to be killed, please confirm how they will be killed and name the appropriately trained person who will perform this:

They will be frozen at -80˚C by Lucy Nevard.
B10. Where will the research be conducted: In the Vallejo-Marin lab (3B161, Cottrell Building) at the University of Stirling.
B11. Have the suitability and availability of the required facilities been confirmed by the relevant NACWO: **Yes** ☐ **No** ☐

SECTION C: Experimental protocols

C1. Does this application involve ASPA regulated procedures?	**Yes** ☐ **No** ☒

C2. If YES please specify the Project Licence Holder who has agreed to be responsible and their licence number:

Project Licence Holder: Click here to enter name **Licence number:** Click here to enter number

C3. If YES please detail the protocols, the Personal Licence Holder competent to perform them and their licence number. The Personal Licence Holder with primary responsibility must be listed first.

Protocol No: Click her to enter number **Description:** Click here to enter description
PIL Name: Click here to enter text **PIL Number:** Click here to enter text

Protocol No: Click her to enter number **Description:** Click here to enter description
PIL Name: Click here to enter text **PIL Number:** Click here to enter text

Protocol No: Click her to enter number **Description:** Click here to enter description
PIL Name: Click here to enter name **PIL Number:** Click here to enter number

If further protocols are involved, copy and paste the above fields as required.

SECTION D: Research involving human participants

D1. Does your research involve human participants? e.g. interviews, questionnaires, telephone surveys	**Yes** ☐ **No** ☒

If YES please also submit an application to GUEP (click here STAFF / UG / TPG) – these applications can run in parallel.

Click here to enter text

SECTION E: Data protection, copyright and other considerations

E1. Does the proposed research involve accessing records of personal or confidential information?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E2. Does the proposed research involve the recording or use of audio-visual material for which consent is required? *Answer **NO** if you are making use of material for which appropriate consent has already been gained e.g. utilisation of established databases*	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E3. Does the proposed research involve the remote acquisition of data from or about human participants using the internet and its associated technologies	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E4. Does the proposed research involve accessing potentially sensitive data through third parties?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E5. Does the proposed research involve reproducing copyrighted work in published form (other than brief citation)?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E6. Does the proposed work involve activities which could temporarily or permanently damage or disturb the environment, or archaeological remains and artefacts?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E7. Does the proposed work involve a potential conflict of interest or raise ethical issues regarding the source of funding or where publication of research data may be restricted?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E8. Has the project been subject to any external ethical review process?	**Yes** ☐ **No** ☒
If YES please give details: **Click here to enter text**	

Please confirm that you have read and understood the University's guidance on GDPR and that the necessary steps have been considered to protect the data of the participants of your research.
Yes ☐ Not Applicable ☐

By signing below (digital signatures accepted), you certify that the information provided is true and correct to the best of your knowledge

STUDENTS

Applicant's signature: **Lucy Nevard** **Date:** 22/06/2018

Supervisor's signature: **Mario Vallejo-Marín** **Date:** 22/06/2018

Approver	Date	Comments
A Elliott (Lay Chair)	17/07/2018	No concerns
J Turnbull (Deputy Chair)		
K Willoughby (HO)		
J Baily (NVS)		
A Pargana (NVS)	09/07/2018	No concerns
B Craig (NACWO)	24/07/2018	No concerns
C Forrest (NACWO)	10/07/2018	No issues
C Matthews (NACWO)		
A McPhee (NACWO)		
K Ranson (NACWO)		
C Bull (Scientific Member)		
P Dudchenko (Scientific Member)		
K Howie (Lay Statistician)		
D Little (Scientist without involvement in ASPA)		
D Penman (Scientific Member)		
A Preston (Scientific Member)		

ASPA Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member, 1 x NACWO, NVS
Non ASPA Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member
Licence Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member, 2 x NACWO, NVS
Retrospective Review requires a minumum of both Chairs and NVS

Signature page: Received
Submission date: 25/06/2018
Approval date: 09/08/2018

Appendix B: Ethics form for Chapter Five

Animal Welfare and Ethical Review Body (AWERB)

Research Proposal Ethical Approval Form

SECTION A: Applicant details

A1. Name of applicant (principal researcher/student): Lucy Nevard
A2. Email address: lucy.nevard1@stir.ac.uk
A3. Faculty affiliation: Natural Sciences **Division/Research group: BES**
A4. Designation: Research Postgraduate
A5. STUDENTS ONLY **Course Name:** PhD Researcher **Academic Supervisor responsible for procedures:** Mario Vallejo-Marin
A6. RESEARCH STAFF ONLY **Job title:** PhD Researcher
A7. Type of review required **ASPA ☐** **Non-ASPA ☒**
A8. Has a health and safety risk assessment been successfully completed **Yes ☒** **No ☐ Not Applicable ☐**

FOR ADMINISTRATIVE USE ONLY: **Ethics application reference numbers: AWERB/2021/204** **Application complete, signed and dated ☒** **Email confirmation**
AWERB decision **Date 24/08/2020** **Approved ☒** **Approved subject to minor amendments ☐** **Rejected ☐** **Deferred ☐**

Reason for rejection/details of required amendments:

Click here to enter text

SECTION B: Project/protocol details

B1. Project/protocol title: Floral Volatile Extraction and Bee Behavioural Assay

B2. Project/protocol funding source: Research Council (RES)
If 'other' please specify: NERC IAPETUS funded PhD

B3. Proposed project/protocol start date: 24/08/2020	**End date:** 30/02/2021

B4. Short project/protocol description including the maximum length of the project:

This project will investigate the effect of floral orientation (i.e. manipulated to face upwards or sideways) on pollen transfer by bumblebees within different species of Solanum.

Experiments will take place in a large flight arena in the lab. Bees will be exposed to flowers (Solanum spp.) grown in the university glasshouses. Some flowers will have pollen tagged with quantum dots, which are non-toxic tags which cause pollen to fluoresce under UV light. Bees will be allowed to forage individually on flowers for up to 30 minutes per trial. Bees will either visit one flower and gather pollen, and then will be captured to look at pollen placement on the bee body, or they will visit two flowers (transferring pollen from one flower to the other) and then will be captured. Behaviour will be recorded by the observer (Lucy Nevard) and using a video camera. Bees used in trials will not return to the colony and will be captured after trials and euthanised by freezing at -80˚C. At the end of the experiment, all bees will be euthanised by freezing at -80˚C.

Maintenance of bumblebee colonies

Captive native bumblebee colonies (Bombus terrestris audax) will be sourced from Biobest (a commercial company providing bumblebees for pollination) and kept in the lab under established conditions re lighting and feeding.

These are imported and commercially reared animals, so they must be euthanised at the end of the experiment to prevent accidental release to the environment. The Bumble Bee Conservation Trust advocates that steps should be taken to avoid the accidental release of imported bees including commercial B. terrestris audax.

The project will last up to 6 months maximum, but these experiments are expected to take around 2 months.

B5. Provide a brief justification for the proposed study:

Buzz pollination (insect vibration of the flower to release pollen) is required for over 22,000 plant species, including economically important crops within *Solanum* (tomatoes, aubergines, etc.) However, the effect of floral morphology and orientation on bee handling on the flower and pollen transfer to other flowers is poorly understood. Many *Solanum* species are oriented downwards, whilst some species with distinct morphologies are oriented sideways. These experiments will give insight into the effect of floral orientation on pollen transfer in different *Solanum* species.

B6. What are the study's main objectives?

To investigate the effect of floral orientation on pollinator behaviour and pollen transfer.

B7. Provide details of the experimental design, the number of animals to be used and the statistical power of the proposed study, demonstrating that the benefits of the experiment outweigh the costs to the animal(s) involved:

In each round of experiments, two bumble bee colonies will be used, each containing approximately 40 workers.

Multiple colonies are necessary to account for any between-colony variation. Linear mixed effect models will be used for statistical analysis, allowing us to account for individual- and colony-level variation. The number of bees, colonies and statistical approach is based on similar experiments conducted by previous undergraduate and post-graduate researchers in the Vallejo-Marin Lab, and published in the primary literature (e.g. Solis-Montero and Vallejo-Marin. 2017. Ecology and Evolution; Morgan et al. 2015. Journal of Insect Behavior; De Luca et al. 2015. Naturwissenschaften; Arroyo-Correa et al. 2019. Journal of Experimental Biology).

We expect to use a maximum of 6 bumblebee colonies.

B8. How have the 3Rs been addressed?

Replacement: Live bees are required to study plant-pollinator interactions.

Reduction: The number of colonies to be used represents the trade-off between replication needed for statistical accuracy and utilising the fewest animals possible.

Refinement: The Vallejo-Marin Lab has conducted similar experiments for over 10 years and follows a methodology that minimises the distress to the bees.

B9a. If animals subject to ASPA regulations are to be killed, please confirm that this will be by a Schedule 1 method and name the appropriately trained person who will perform this:

N/A

B9b. If animals not subject to ASPA regulations are to be killed, please confirm how they will be killed and name the appropriately trained person who will perform this:

The captive bumblebees will be humanely euthanised at -80˚C by Lucy Nevard.

B10. Where will the research be conducted (information on Insurance for staff and students can be found here) :

University of Stirling

B11. Have the suitability and availability of the required facilities been confirmed by the relevant NACWO:

Yes ☐ No ☐ Not Applicable ☒

SECTION C: Experimental protocols

C1. Does this application involve ASPA regulated procedures?	**Yes ☐ No ☒**

C2. If YES please specify the Project Licence Holder who has agreed to be responsible and their licence number:

a) **Project Licence Holder:** Click here to enter name **Licence number:** Click here to enter number

b) **Copy the relevant protocols and steps from the Project Licence under which this work will be conducted.**
Click or tap here to enter text.

C3. If YES please detail the regulated procedures associated with relevant steps in the protocol above an name the Personal Licence Holder(s) signed off as competent to perform them giving their personal licence number(s). The Personal Licence Holder with primary responsibility must be listed first.

Step No: Click her to enter number **Associated Regulated Procedure:** Click here to enter description
Competent PIL Name: Click here to enter text **PIL Number:** Click here to enter text

Step No: Click her to enter number **Associated Regulated Procedure:** Click here to enter description
Competent PIL Name: Click here to enter text **PIL Number:** Click here to enter text

If further protocols are involved, copy and paste the above fields as required.

SECTION D: Research involving human participants

D1. Does your research involve human participants? e.g. interviews, questionnaires, telephone surveys	**Yes ☐ No ☒**

If YES please also submit an application to GUEP – these applications can run in parallel.
Click here to enter text

SECTION E: Data protection, copyright and other considerations

E1. Does the proposed research involve accessing records of personal or confidential information?	**Yes ☐ No ☒**

If YES please give details: Click here to enter text	
E2. Does the proposed research involve the recording or use of audio-visual material for which consent is required? *Answer **NO** if you are making use of material for which appropriate consent has already been gained e.g. utilisation of established databases*	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
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If YES please give details: Click here to enter text	
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If YES please give details: Click here to enter text	
E6. Does the proposed work involve activities which could temporarily or permanently damage or disturb the environment, or archaeological remains and artefacts?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E7. Does the proposed work involve a potential conflict of interest or raise ethical issues regarding the source of funding or where publication of research data may be restricted?	**Yes** ☐ **No** ☒
If YES please give details: Click here to enter text	
E8. Has the project been subject to any external ethical review process?	**Yes** ☐ **No** ☒
If YES please give details: **Click here to enter text**	

Please confirm that you have read and understood the University's guidance on GDPR and that the necessary steps have been considered to protect the data of the participants of your research.

Yes ☐ **Not Applicable** ☒

By signing below, you certify that the information provided is true and correct to the best of your knowledge

STUDENTS

Applicant's signature: Lucy Nevard (electronic signature) Date: 18/08/2020

Supervisor's signature: Mario Vallejo-Marin Date: 19/08/2020

Applicant: Lucy Nevard/ Mario Vallejo-Marin AWERB (20 21) 204 Non ASPA

Approver	Date	Comments	Suggested Outcome Code
A Elliott (Lay Chair)			
J Turnbull (Deputy Chair)(NVS)	21/08/2020	No ethical concerns	A
M Tomlinson (HO)			
J Baily (NVS)			
B Craig (NACWO)			
C Forrest (NACWO)			
A Powell (NACWO)			
K Ranson (NACWO)			
A Law (Scientific Member)			
S Kessler (Scientific Member)	21/08/2020	No ethical concerns	A
B Glencross (Scientific Member)			
A Desbois (Scientific Member)			

ASPA Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member, 1 x NACWO, NVS
Non ASPA Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member
Licence Approval requires a minimum of : Chair / Deputy Chair, 1 x Scientific Member, 2 x NACWO, NVS
Retrospective Review requires a minumum of both Chairs and NVS

Signature page:	Y	Email
Submission date:	19/08/2020	
Approval date:	24/08/2020	