

Variation in the natural frequency of stamens in six morphologically diverse, buzz-pollinated, heterantherous *Solanum* taxa and its relationship to bee vibrations

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During buzz pollination, bees use vibrations to remove pollen from flowers. Vibrations at the natural frequency of pollen-carrying stamens are amplified through resonance, resulting in higher amplitude vibrations. Because pollen release depends on vibration amplitude, bees could increase pollen removal by vibrating at the natural frequency of stamens. However, few studies have characterized the natural frequencies of stamens and compared them to the frequencies of buzz-pollinating bees. We use laser Doppler vibrometry to characterize natural frequencies of stamens of six morphologically diverse, buzz-pollinated, heterantherous *Solanum* taxa and compare the frequency of bumblebee buzzes produced on two *Solanum* spp. with different natural frequencies. We found that stamen morphology and plant identity explain variation in their natural frequency. The natural frequencies of the stamens in the studied *Solanum* taxa fell between 45 and 295 Hz; in five out of six taxa the frequencies were < 190 Hz, which only partly overlaps floral vibrations of buzz-pollinating bees. We show that captive bumblebees produce vibrations at a frequency of 345 Hz and do not change their floral vibrations to match the natural frequency of the visited flowers. Our results suggest that pollen release induced by vibrating stamens at their natural frequencies might only play a role in a subset of buzz pollination interactions.

ADDITIONAL KEYWORDS: biomechanics – *Bombus terrestris* – buffalo-bur – buzz pollination – flower diversity – nightshade – resonance – Solanaceae – *Solanum citrullifolium* – *Solanum rostratum*.

INTRODUCTION

More than half of all bee species have evolved the ability to vibrate to extract pollen from flowers, giving rise to the syndrome of buzz pollination (Buchmann, 1983; Cardinal, Buchmann & Russell, 2018; Vallejo-Marín, 2019). Most buzz-pollinated flowers present evolutionarily derived morphologies in which pollen locked inside stamens is released through small pores (poricidal stamens) (Buchmann, 1983). While buzzing flowers, bees hold stamens using their mandibles and legs and activate their thoracic muscles (De Luca & Vallejo-Marín, 2013; Fig. 1A). Pollen release from poricidal stamens is a function of the vibration characteristics, mainly its amplitude (Harder & Barclay, 1994; King & Buchmann, 1996; De Luca *et al.*, 2013; Kemp & Vallejo-Marín, 2020; Rosi-Denadai *et al.*, 2020).

The amplitude of a floral vibration depends on the characteristics of the bee (King & Buchmann, 2003), the coupling between bee and flower (King, 1993; Arroyo-Correa, Beattie & Vallejo-Marín, 2019), and the vibrational properties of the stamen (anther and filament) (Buchmann & Hurley, 1978; Mortimer, 2017; Vallejo-Marín, 2019; Brito *et al.*, 2020).

One vibrational property of solid structures, including stamens, is the natural frequency. Natural frequencies are the frequencies at which objects vibrate when disturbed, and are given by their mass, shape and material properties, such as rigidity or stiffness (Volterra & Zachmanoglou, 1965; Niklas, 1992). When a structure is vibrated at its natural frequency it resonates, causing higher amplitude vibrations. The first natural frequency is the lowest frequency at which an object resonates. Complex systems can have more than one natural frequency (Volterra & Zachmanoglou, 1965; Niklas, 1992). Stamens may behave analogously to a cantilever beam

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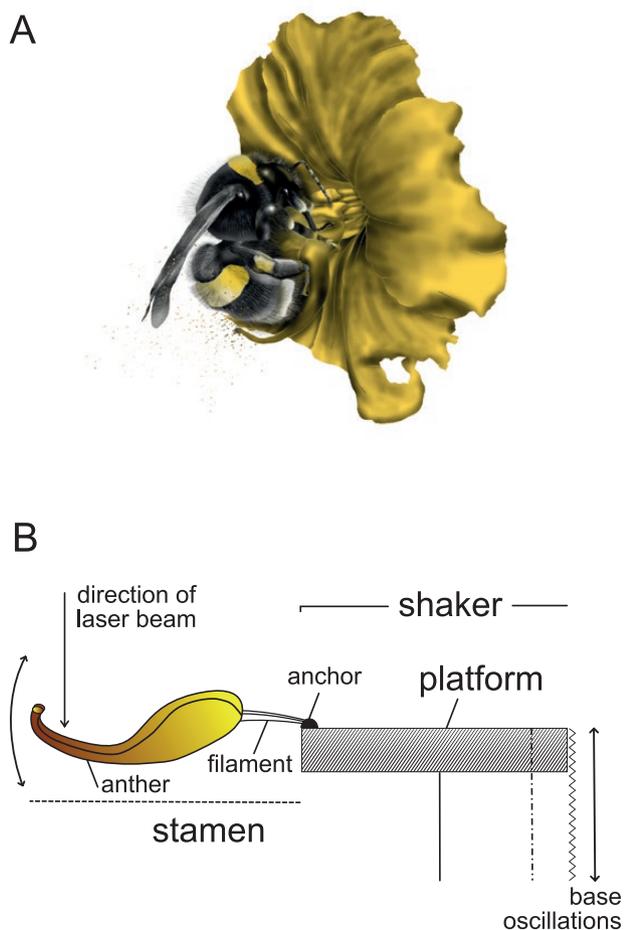


Figure 1. A, illustration showing *Bombus terrestris* vibrating the stamens of buzz-pollinated *Solanum rostratum*. B, diagram of the experimental setup showing the stamen attached to a platform on the magnetic shaker, the direction of the base oscillations and the position of the laser beam of the Doppler vibrometer.

(King & Buchmann, 1995), a structure fixed at one end and free at the other, which has multiple normal modes (Fletcher 1992). The first normal mode, or natural frequency, of a cantilever beam-like structure should correspond to the highest achievable amplitude at resonance (Volterra & Zachmanoglou, 1965). In principle, if the vibrations applied by bees occurred at the natural frequency of stamens, vibration amplitude would increase through resonance, resulting in higher pollen removal (King & Buchmann, 1996; Timerman & Barrett, 2019).

The relevance of stamen natural frequencies for pollen release is linked to the type of vibrations that bees can produce. In bees, the fundamental frequency of floral vibrations is distinct (higher than) from vibrations produced in other behaviours such as flight or defence (Pritchard & Vallejo-Marín, 2020).

Previous work on the spectral properties of buzz pollination has established that the fundamental frequency of bee vibrations on flowers varies across bee species and ranges from ~100 to 400 Hz with significant variation within and among bee taxa (De Luca & Vallejo-Marín 2013; Corbet & Huang, 2014; Switzer & Combes, 2017; De Luca *et al.*, 2019; Pritchard & Vallejo-Marín, 2020; Rosi-Denadai *et al.*, 2020). Unlike the frequency of flight vibrations, which varies negatively with individual size, the variation in the fundamental frequency of floral vibrations across bee species is not strongly associated with size across species (De Luca *et al.*, 2019). Bees may use vibrations of different frequency when visiting different species of buzz-pollinated flowers (Corbet & Huang, 2014; Switzer & Combes, 2017). However, experimental studies with captive bumblebees have not found large changes in buzz frequency when the same bee species visits different flower species (Arroyo-Correa *et al.*, 2019) or when bees are trained in artificial flowers that release pollen at different specific frequencies (Switzer *et al.*, 2019). Regardless of whether bees can adjust their vibration frequency to match the flowers that they visit, it is unclear whether the frequencies of floral vibrations caused by bees overlap the natural frequencies of stamens.

In contrast to the numerous studies on the spectral properties of buzz-pollinating bees, to date, little is known about the natural frequencies of stamens of buzz-pollinated plants. A pioneer study by King & Buchmann (1996) found that the natural frequency of stamens of *Solanum laciniatum* Aiton (Solanaceae) was significantly lower (124 Hz) than the fundamental frequencies of bees buzzing these flowers (316 Hz). Other studies on the natural frequencies of flowers have focused on wind-pollinated plants, in which vibrations induced by air flow lead to pollen ejection (Timerman *et al.*, 2014; Timerman & Barrett, 2018, 2019). Further work is needed to document the natural frequencies of other buzz-pollinated flowers and compare them to the types of vibrations produced by bees.

Here, we exploit natural variation both between and within plant species to investigate the natural frequency of buzz-pollinated flowers. We use an unusual group of taxa of *Solanum* L. showing repeated independent transitions in flower and stamen morphology (Vallejo-Marín *et al.*, 2014). Unlike most *Solanum* spp. (Särkinen *et al.*, 2013), taxa in *Solanum* section *Androceras* Whalen are heterantherous, bearing two sets of stamens with different morphologies specialized in attracting and rewarding pollinators (feeding stamens) or fertilization (pollinating stamens) (Müller, 1881; Vallejo-Marín *et al.*, 2009). The flowers of *Solanum* section *Androceras* studied here have four smaller stamens, located towards the centre of the flower (feeding stamens), and a single stamen, usually

large and curved and located away from the centre of the corolla (pollinating stamen) (Whalen, 1978, 1979; Vallejo-Marín *et al.*, 2009; Fig. 1A). We study three pairs of closely related taxa in which one member is large-flowered and highly heterantherous, with larger, more conspicuous pollinating anthers, and the other is small-flowered and less heterantherous, with smaller, less conspicuous pollinating anthers (Whalen, 1978, 1979; Stern, Weese & Bohs, 2010; Vallejo-Marín *et al.*, 2014). This combination of (i) within-flower variation in stamen morphology in heterantherous flowers and (ii) phylogenetically independent transitions in floral form provides a system to investigate variation in the natural frequencies in buzz-pollinated flowers. Our study addresses two questions. (1) To what extent do stamens with different morphologies have different natural frequencies? (2) Do bumblebees dynamically adjust the frequency of their vibrations while visiting flowers that differ in the natural frequency of their stamens?

MATERIAL AND METHODS

PLANT STUDY SYSTEM

We studied six taxa of *Solanum* section *Androceras*, native to Mexico and the southern USA, that comprise three pairs of closely related or sister taxa in each of the three series of the section (see Supporting Information Table S1 for accession information): *S. fructu-tecto* Cav. and *S. rostratum* Dunal of series *Androceras* Whalen; *S. citrullifolium* A. Braun and *S. heterodoxum* Dunal of series *Violaceiflorum* Whalen; and *S. grayi* Whalen var. *grandiflorum* Whalen and *S. grayi* var. *grayi* of series *Pacificum* Whalen (Whalen, 1979; Stern *et al.*, 2010). As in other wild *Solanum* spp., the pollinators of *Solanum* section *Androceras* include buzz-pollinating bees of a varied range of sizes including bumblebees, *Bombus* spp., which have been observed on *S. rostratum* and *S. angustifolium* Mill., and other medium-sized bees (*Centris* spp.) observed on *S. grayi* var. *grandiflorum* and *S. lumholtzianum* Barlett (M.V.M., pers. obs.). Published studies on the pollination ecology of *Solanum* section *Androceras* have mainly focused on the widely distributed *Solanum rostratum*. This species is pollinated by diverse buzz-pollinating visitors, including small bees (e.g. *Augochloropsis*, *Exomalopsis*, *Lasioglossum*) and medium to large bees (e.g. *Bombus*, *Centris*, *Thygater*, *Xylocopa*) (Bowers, 1975; Solís-Montero, Vergara & Vallejo-Marín, 2015; Solís-Montero *et al.*, 2018). In its introduced range in China, *S. rostratum* is also visited by similar bees including *Halictus*, *Bombus* and *Xylocopa* (Zhang & Lou, 2015).

For this study, plants were germinated from seeds previously collected in the field (all taxa except *S. citrullifolium*) or obtained from the Solanaceae collection previously kept at the Radboud Botanic Gardens (*S. citrullifolium*; see Supporting Information Table S1 for source and accession numbers). Seed germination and plant growth were carried out at the University of Stirling plant growth facilities. Briefly, seeds were germinated following a 24-h treatment with 1000 p.p.m. gibberellic acid (GA3; Sigma-Aldrich, Dorset, UK) in aqueous solution. Germinated seedlings were transplanted after 2–3 weeks to 1.5-L pots containing a mix of All Purpose Growing Medium and Perlite Standard (4:1; William Sinclair Horticulture PLC, Lincoln, UK), and fertilized weekly with Tomorite Concentrated Tomato Food (Levington, Surrey, UK). Supplemental light was provided by compact fluorescent lamps for 16 h per day and supplemental heating was provided to maintain minimum temperatures at 16 and 25 °C (night and day, respectively). A subset of plants was transplanted 2–3 weeks later to a large bench with the same soil mix (approx. 5 m × 1 m × 70 cm) with plants spaced 60 cm apart, to encourage flowering. The large benches had supplemental heating but not supplemental light.

NATURAL FREQUENCY OF STAMENS

Vibration measurements were done in a laboratory with controlled temperature and humidity (21 °C; 60% relative humidity). Flowers for the experiment were collected in the morning of each measurement day, from 08:00 to 09:00 h, by cutting entire inflorescences and placing the inflorescence stalk in water. We only used unvisited flowers that opened on the same day as the measurements were taken. In these species, poricidal anthers are dehiscent upon anthesis. We used a single stamen cut at the base of the filament where it connects with the receptacle and measured two stamens from each flower, one feeding and one pollinating. Stamens cut from flowers were kept inside a plastic container lined with humid paper towels until ready to be measured. Measurements from cut stamens were taken as quickly as possible to avoid desiccation and potential changes in the material properties of the stamens.

To measure natural frequencies, we estimated frequency spectra of stamens exposed to broadband white noise (King, 1993; King & Buchmann, 1995, 1996, 2003). Single stamens were exposed to white noise vibrations (a randomly generated mix of frequencies between 20 and 20 000 Hz) generated in *Audacity* (v.2.4.1, Audacity Team, 2019), using a linear power amplifier (LDS-LPA100, Brüel & Kjær, Nærum, Denmark) and a permanent magnetic shaker platform (LDV210, Brüel & Kjær). Each stamen was glued

(Loctite Ultra Gel Control, Henkel, Hemel Hempstead, UK) by its filament base to a rigid platform at the top of the shaker (Fig. 1B). As even small changes in mass might affect their dynamic properties, we applied low accelerations which were not sufficient to remove pollen from flowers and ensured that the mass of the flower remained constant throughout each measurement.

We measured the vibration response of stamens using a laser Doppler vibrometer, which uses the Doppler effect of a laser beam reflected on a target surface to estimate vibrational properties, without the need for physical contact between the measuring equipment and the target. We used a PDV-100 laser Doppler vibrometer (Polytec, Waldbronn, Germany) set to 500 mm s⁻¹ sensitivity, a low-pass filter of 5 kHz and no high-pass filter. We focused the laser beam as close to the apical end of the stamen as possible at an axis perpendicular to the stamen length, parallel to the main axis of displacement of the shaker platform (Fig. 1B). An accelerometer (0.8 g, 352A24, PCB Piezotronics, Depew, NY, USA) was attached to the shaker to record reference measurements. The laser vibrometer (recorded in acceleration units) and the accelerometer signals were simultaneously acquired using VibSoft-20 (Polytec) at a sampling rate of 12 000 samples per second, using a 20–5000-Hz bandpass filter, and recorded for 1.28 s (15 360 samples; resolution 0.781 Hz). We obtained the frequency spectra in the range from 20 to 2500 Hz using a fast Fourier transform (FFT; 6375 lines with a Hamming window) using VibSoft-20 and calculated the average frequency spectrum of ten replicate measurements for each stamen.

To estimate the first natural frequency (hereafter natural frequency), we visually identified the first (lowest frequency) peak in the frequency spectrum (range 20–600 Hz) and obtained its associated frequency. This value corresponds to the first natural frequency (King & Buchmann, 1996; Timerman *et al.*, 2014). The first natural frequency in a cantilever beam is expected to be associated with the highest resonance amplitude (Volterra & Zachmanoglou, 1965). Natural frequencies were assessed in an average of nine flowers per taxon (range five to 11, $N = 54$ flowers) from two to eight individuals per taxon (two for *S. fructu-tecto*; average of 1.46 flowers per individual, $N = 41$ individuals; Supporting Information Table S1).

To characterize floral morphology, we measured 11 stamen and floral traits and calculated two others from those. These 13 floral traits were measured for the same 54 flowers used to calculate natural frequencies to allow us to establish correlations among traits that could influence natural frequencies. The measured traits were flower mass, corolla height, corolla width,

stamen length, anther length, anther major diameter, anther minor diameter, filament major diameter, filament minor diameter, stamen mass and anther mass. These traits were measured separately for pollinating and feeding stamens. Filament lengths and filament masses were calculated from the above measurements.

FREQUENCY OF FLORAL VIBRATIONS USED BY BEES ON TWO PLANT SPECIES WITH CONTRASTING MORPHOLOGIES

We compared the floral vibrations produced by captive bumblebees while visiting two plant species with contrasting floral morphologies: *S. citrullifolium* and *S. heterodoxum*. These two plant species are closely related, but they differ strongly in their floral morphology and in the first natural frequencies of their stamens (see Results). *Solanum citrullifolium* has relatively large flowers, and the two anther types are well differentiated with the single pollinating anther being larger in size, S-shaped and violet-coloured, in contrast to the four smaller, straight-shaped, yellow-coloured feeding anthers (Vallejo-Marín *et al.*, 2014). *Solanum citrullifolium* has flowers of similar size or larger than the bumblebee-pollinated flowers of *S. rostratum* and is probably visited by similarly sized pollinators, although we have not been able to locate published accounts of its pollination ecology. In contrast, the flowers of *S. heterodoxum* are much smaller, with smaller anthers and less differentiated anther types, being almost isoantherous (Vallejo-Marín *et al.*, 2014); this species sets abundant fruits in the absence of pollinators (our observations). The small size of its anthers might make its flowers difficult to manipulate by large-bodied bumblebees.

We used one colony of *Bombus terrestris* subsp. *audax* (Biobest, Westerlo, Belgium; hereafter *B. terrestris*). We provided the colony with sugar solution (Biobest) *ad libitum*. The colony was attached to a flight arena (122 × 100 × 37 cm), illuminated with an LED light panel (59.5 × 59.5 cm, 48-W Daylight; Opus Lighting Technology, Birmingham, UK) and maintained on a 12:12-h supplemental dark:light cycle. Room temperature was 22–23 °C and relative humidity was 50–60%. Although *B. terrestris* is native to Europe and hence not a natural pollinator of *Solanum* section *Androceras*, we considered this bee species a useful model to study bee vibrations on buzz-pollinated flowers as the *Solanum* taxa studied are pollinated by buzzing bees of similar size, including bumblebees, in their native range (Solís-Montero *et al.*, 2015).

We placed a single flower of either *S. citrullifolium* or *S. heterodoxum* in the flight cage, allowing a bee to forage freely for ~10 min (visitation bout). We

recorded up to 3 min of floral buzzes using a digital audio recorder with two unidirectional condenser microphones (Zoom H4n Pro Handy, Zoom North America, Hauppauge, NY, USA) placed always at 10 cm from the flower, sampling the audible component of floral vibrations at 48-kHz sampling rate. This is a well-established and effective method for recording the frequency component of floral vibrations caused by bees (De Luca *et al.*, 2018). Fresh flowers were used for each bout. Naïve bees, i.e. bees with no previous experience of foraging on flowers, were first exposed to *S. citrullifolium* for six consecutive visitation bouts ($N = 10$ bees), and buzzes in the first and sixth bout were analysed ($N = 1640$ buzzes analysed). Then, the same bees were exposed to *S. heterodoxum* for six additional bouts and buzzes in the first ($N = 10$ bees) and sixth bouts ($N = 3$ bees) were analysed ($N = 758$ buzzes). The lack of a reciprocal treatment (*S. heterodoxum*, then *S. citrullifolium*) was due to the reluctance of naïve *B. terrestris* to visit the small-flowered taxon (naïve bees readily visit *S. citrullifolium*). To obtain the fundamental frequency of the floral vibrations produced by bumblebees on *Solanum* flowers, we used *Audacity* to obtain the frequency spectrum (FFT) of each floral buzz using a Hamming window (size = 512) and visually identified the fundamental frequency (Morgan *et al.*, 2016).

STATISTICAL ANALYSES

We estimated the correlation among 13 floral traits and natural frequency using Pearson's correlations. We calculated separate correlation matrices for each stamen type (feeding and pollinating) and visualized the results using the package *corrplot* (Friendly, 2002). To analyse variation in the natural frequency and stamen characteristics, we fitted a series of linear mixed-effects models with natural frequency as the response variable, stamen length, stamen type and relative flower size (large or small) as fixed effects, and plant taxon ('species') as a random effect using *lme4* (Bates *et al.*, 2015). Stamen length was chosen for analysis because it was strongly and positively correlated with all other floral traits (see Results). Model selection was carried out by starting with a model that contained all terms plus the interaction 'stamen type × relative flower size' and sequentially removing non-significant terms as assessed by a likelihood ratio test (LRT). The final selected model included both anther length and stamen type. Statistical significance of fixed effects in the final model was assessed with F-statistics with Satterthwaite correction for degrees of freedom, implemented in *lmerTest* (Kuznetsova, Brockhoff & Christensen, 2017).

To analyse the differences among the fundamental frequencies of bees, we also fitted a linear mixed-effects model with plant species and bout number as fixed effects, and individual bee identity as a random effect. All analyses were done in *R* v.4.0.2 (R Development Core Team, 2020).

RESULTS

NATURAL FREQUENCIES OF STAMENS

Examples of the frequency spectra for feeding and pollinating stamens of two species are shown in Figure 2. Morphological and mass measurements of the 13 floral traits studied here are summarized in Supporting Information Table S2. All measured traits of flower morphology and mass were positively correlated with one another across all *Solanum* taxa studied (Fig. 3). The natural frequency of both feeding and pollinating stamens was negatively correlated with all measured floral traits (Pearson's $\rho < -0.11$ for all traits; Fig. 3), except in the case of pollinating stamens, which showed a weak positive correlation between frequency and filament major diameter ($\rho = 0.05$; Fig. 3). Stamen length was strongly correlated with all other floral traits in both feeding and pollinating stamens ($\rho = 0.37-0.95$; Fig. 3; the numerical values for each correlation are shown in Fig. S1). Given the strong correlations among floral traits, we used stamen length in the statistical analyses (linear mixed-effects models) between floral characteristics and natural frequencies.

Overall, the average natural frequency of individual stamens across six *Solanum* taxa varied from 44.57 ± 1.36 Hz (mean \pm SE) for pollinating stamens of *S. citrullifolium* to 294.30 ± 47.37 Hz for the feeding stamens of *S. grayi* var. *grayi* (Table 1; Fig. 4). Independently of anther type, stamens of large-flowered taxa (*S. rostratum*, *S. grayi* var. *grandiflorum* and *S. citrullifolium*) had on average lower natural frequencies than their closely related paired taxon with smaller flowers (*S. fructu-tecto*, *S. heterodoxum* and *S. grayi* var. *grayi*) (101.48 ± 20.46 Hz vs. 162.33 ± 25.71 Hz, for large- and small-flowered taxa, respectively), although this difference was not statistically significant as assessed by an LRT of nested models with and without flower type (large vs. small; $P = 0.916$). The results of the analysis of mixed-effects models including taxon, stamen type and stamen length indicated that stamen type had a significant effect on the natural frequency of stamens. Pollinating stamens had on average lower frequencies than feeding stamens (Table 2; Fig. 4).

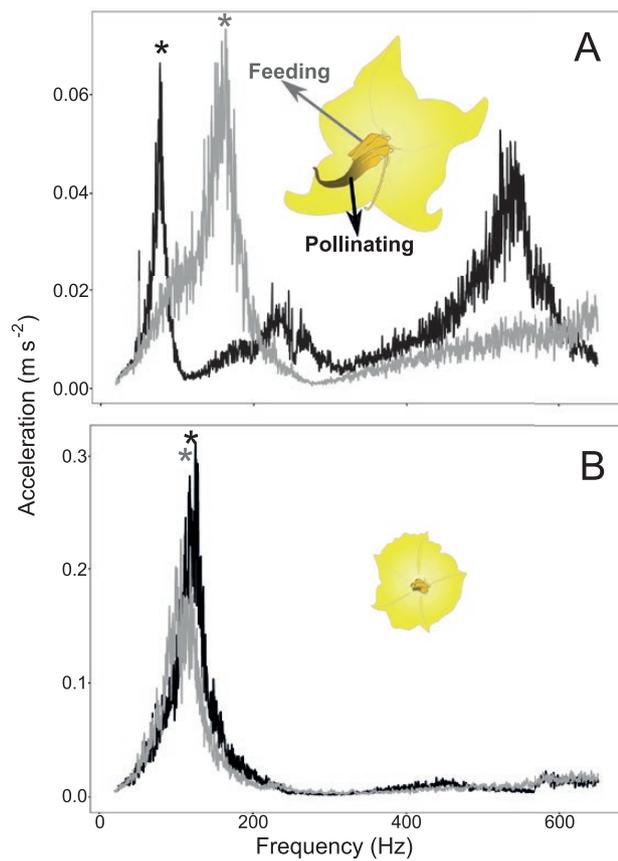


Figure 2. Frequency spectrum obtained in response to the application of broadband frequency vibrations (white noise; 20–20 000 Hz) applied to individual stamens of two buzz-pollinated, heterantherous species of *Solanum* section *Androceras*. The grey lines correspond to feeding stamens and the black lines to pollinating stamens. The feeding and pollinating anthers of *S. rostratum* (A) are morphologically more distinct than those of *S. fructu-tecto* (B), which is weakly heterantherous. The first natural frequency corresponds to the lowest frequency peak observed for each stamen and is indicated for each stamen type with an asterisk.

Differences between pollinating and feeding stamens were more marked in large-flowered taxa and smaller or absent in small-flowered taxa (Table 1; Figs 2, 4), but we did not detect a statistically significant interaction between flower type (large vs. small) and stamen type (feeding vs. pollinating) when comparing nested models using an LRT ($P = 0.693$). For each pair of closely related taxa, pollinating stamens from the large-flowered taxon had lower natural frequencies than pollinating stamens from its small-flowered relative (Fig. 4). Finally, after statistically accounting for species identity and stamen type, we observed a marginally significant negative effect of stamen length on natural frequencies ($P = 0.055$; Table 2). In other

words, longer stamens tended to have lower natural frequencies than shorter stamens (Table 2).

FREQUENCY OF FLORAL VIBRATIONS USED BY BEES ON TWO *SOLANUM* SPECIES WITH CONTRASTING MORPHOLOGIES

We analysed 2398 floral vibrations of ten bees visiting flowers of *S. citrullifolium* and *S. heterodoxum* (1640 and 758 floral vibrations in each plant, respectively). All ten bees visited both plant species at least once, yielding 47–279 buzzes per bee per plant species (164 ± 20.99 and 75.8 ± 14.4 buzzes per bee, for *S. citrullifolium* and *S. heterodoxum*). Two sample floral vibrations of the same individual bee while visiting a flower of each species are shown in Figure 5. We found a statistically significant effect of bout number on the fundamental frequency of floral buzzes (Table 3), but the effect was negligible in *S. citrullifolium* (which was visited first; see Methods). In this case, the frequency of floral vibrations in the first bout was 345.25 ± 0.87 Hz ($N = 10$ bees, 636 buzzes) and 344.04 ± 0.57 Hz in the sixth bout ($N = 10$ bees, 1004 buzzes). The effect of bout was more marked when comparing the first and sixth visit of *B. terrestris* to *S. heterodoxum*. Here, floral buzzes had a fundamental frequency of 349.68 ± 0.70 Hz ($N = 10$ bees, 586 buzzes) in the first visitation bout and 329.47 ± 0.95 Hz ($N = 3$ bees, 163 buzzes) in their sixth bout. We also found, overall, a statistically significant, but biologically minor (~ 5 Hz; Table 3), difference in fundamental frequency among the same ten bees visiting two morphologically distinct flower types, with bees producing on average lower frequency vibrations in flowers of *S. heterodoxum* than in flowers of *S. citrullifolium* (Table 3).

DISCUSSION

In contrast to wind-pollinated plants (Timerman & Barrett, 2018), little is known about the natural frequency of buzz-pollinated flowers, with the notable exception of a study reporting the natural frequency of *Solanum laciniatum* (King & Buchmann, 1996). We have shown here that even closely related taxa in *Solanum* have stamens with different natural frequencies. By studying a closely related group of plants in which a single flower bears two morphologically distinct anthers (heteranthery), we were able to show that the difference in this biomechanical property is also captured within the same flower and that it might be associated with the replicate evolutionary shifts in flower morphology observed in *Solanum* section *Androceras* (Vallejo-Marín *et al.*, 2014). Variation in the natural frequencies of stamens might play a role in regulating patterns of pollen release during buzz

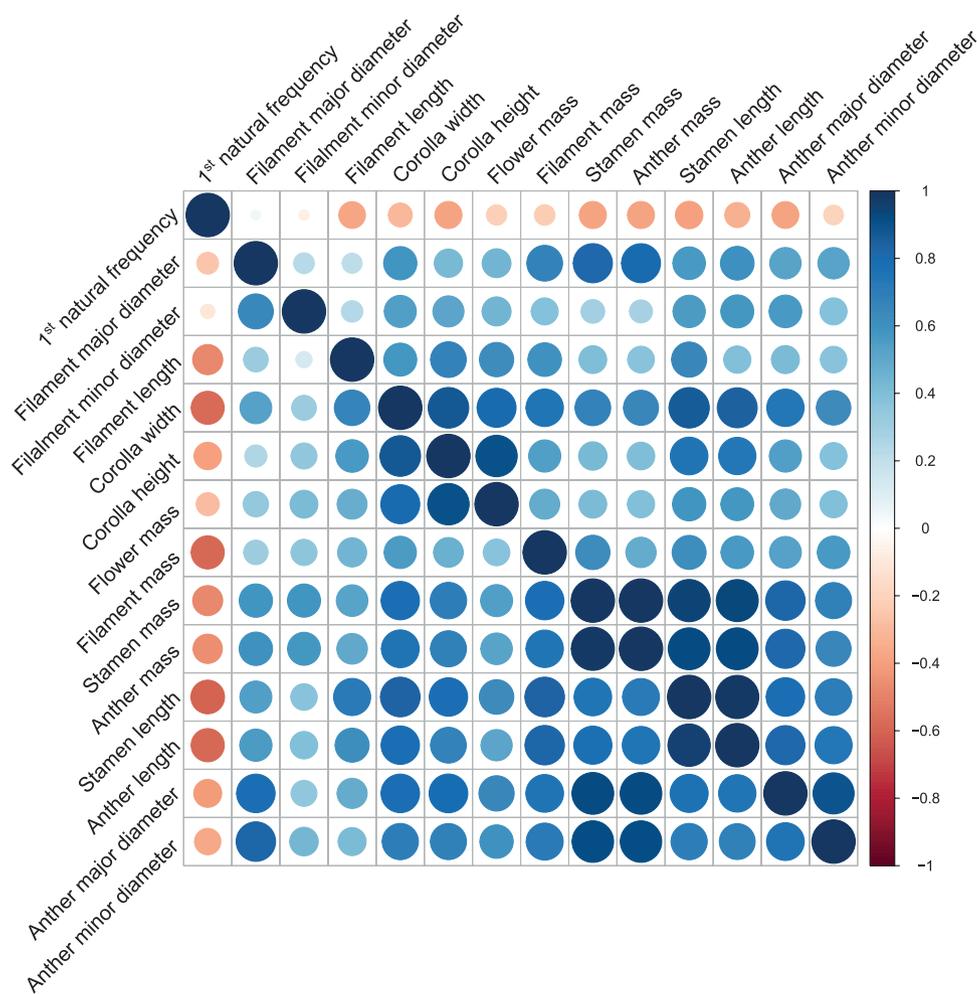


Figure 3. Visual representation of the Pearson product-moment correlation matrix among 13 floral and stamen traits and the natural frequency of stamens across six heterantherous, buzz-pollinated taxa of *Solanum* section *Androceras*. Correlations were calculated separately for pollinating stamens (upper triangle of the matrix) and feeding stamens (lower triangle). Negative correlations are shown in red and positive correlations in blue, with darker colours indicating higher absolute values. The order of the variables shown in the figure was chosen using hierarchical clustering.

Table 1. First natural frequency (in Hz; mean ± SE) of feeding and pollinating stamens of three pairs of heterantherous taxa in *Solanum* section *Androceras*. Each pair of taxa consists of a large-flowered, strongly heterantherous taxon (Large), and a sister-species or closely related taxon with small flowers and weak heteranthery (Small). One anther of each type was analysed per flower

Taxon		Stamen type			
Series	Species	Flower type	Feeding	Pollinating	Number of flowers
<i>Androceras</i>	<i>Solanum rostratum</i>	Large	144.85 ± 17.79	81.14 ± 8.30	11
	<i>S. fructu-tecto</i>	Small	149.40 ± 19.95	117.97 ± 14.94	11
<i>Pacificum</i>	<i>S. grayi</i> var. <i>grandiflorum</i>	Large	189.77 ± 26.65	80.89 ± 6.08	10
	<i>S. grayi</i> var. <i>grayi</i>	Small	294.30 ± 47.37	188.00 ± 30.76	5
<i>Violaceiflorum</i>	<i>S. citrullifolium</i>	Large	64.22 ± 5.00	44.57 ± 1.36	10
	<i>S. heterodoxum</i>	Small	121.82 ± 14.65	120.39 ± 16.92	7

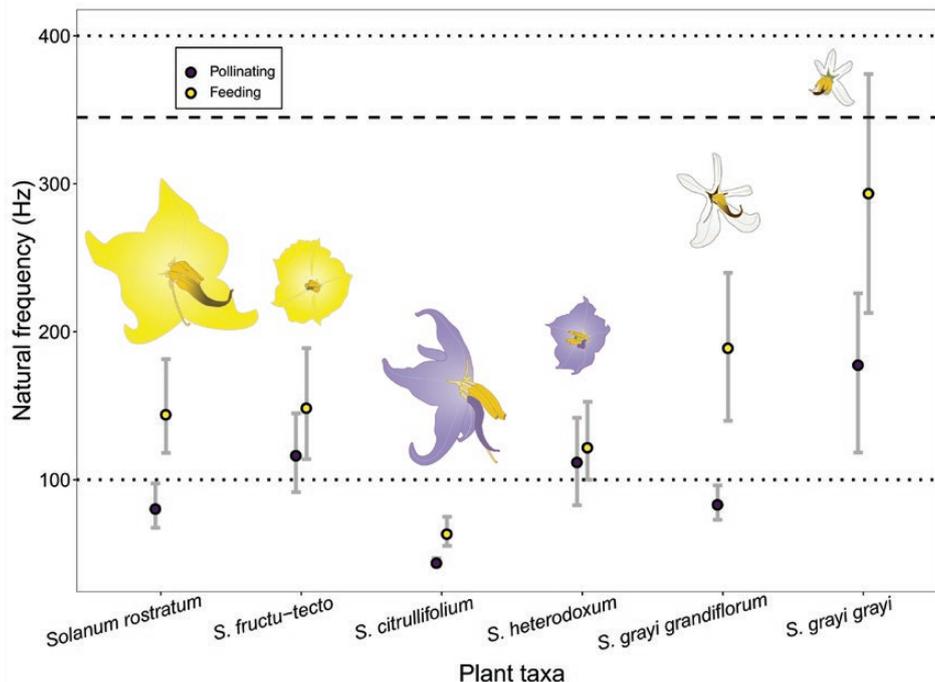


Figure 4. Natural frequencies (mean \pm SE) of feeding and pollinating stamens of three pairs of heterantherous taxa of *Solanum* section *Androceras*. These six taxa represent three independent transitions in flower size, with one large-flowered, highly heterantherous taxon and one small-flowered, weakly heterantherous taxon. Each pair of taxa is associated with a different corolla colour. The average fundamental frequency of floral vibrations produced by *Bombus terrestris* on flowers of *S. citrullifolium* and *S. heterodoxum* obtained in this study is shown with a dashed line. The dotted lines show the range of fundamental frequencies of floral vibrations commonly observed across multiple species of buzz-pollinating bees (100–400 Hz; De Luca & Vallejo-Marín 2013; De Luca *et al.*, 2019). Flower illustrations depict the variation in morphology and preserve size proportions across taxa.

Table 2. Statistical analysis of the effect of stamen type (feeding vs. pollinating) and stamen length (mm) on the natural frequency (Hz) of stamens from three pairs of heterantherous taxa in *Solanum* section *Androceras*. Model estimates were obtained from a linear mixed-effects model with taxon as a random effect and stamen type and stamen length as fixed effects. Statistical significance (*P*-values) of the fixed effects was obtained using type III sums of squares. SE = standard error of the estimate

Model component	Estimate	SE	<i>P</i> -value
Intercept	214.559	34.254	
Stamen type (pollinating stamen)	-37.013	13.712	0.008
Stamen length	-7.598	3.755	0.055

pollination, although further experimental work is needed in this area. Consistent with previous work (Switzer *et al.*, 2019), we found little evidence of rapid adjustments in the frequency of floral vibrations produced by captive bumblebees to match the natural

frequency of the flowers they visit. However, because the natural frequencies of some types of stamens (feeding stamens of five out of six studied species) overlaps the range of fundamental frequencies produced by other bee species, we suggest that stamen resonance might play a role in facilitating pollen release in some buzz pollination systems.

HETERANTHERY AND VARIATION IN NATURAL FREQUENCY

In heterantherous *Solanum*, anther dimorphism is associated with functional specialization of stamens into pollinator attraction and reward (feeding stamens) and fertilization (pollinating stamens) (Vallejo-Marín *et al.*, 2009), and we show here that these functional differences are paralleled by distinct natural frequencies between stamen types. The average natural frequency of the feeding anthers of all taxa, except *S. citrullifolium*, are between 100 and 320 Hz, compared with the range of fundamental frequencies of 100–400 Hz observed across bee species (De Luca & Vallejo-Marín, 2013; Corbet & Huang, 2014; De Luca

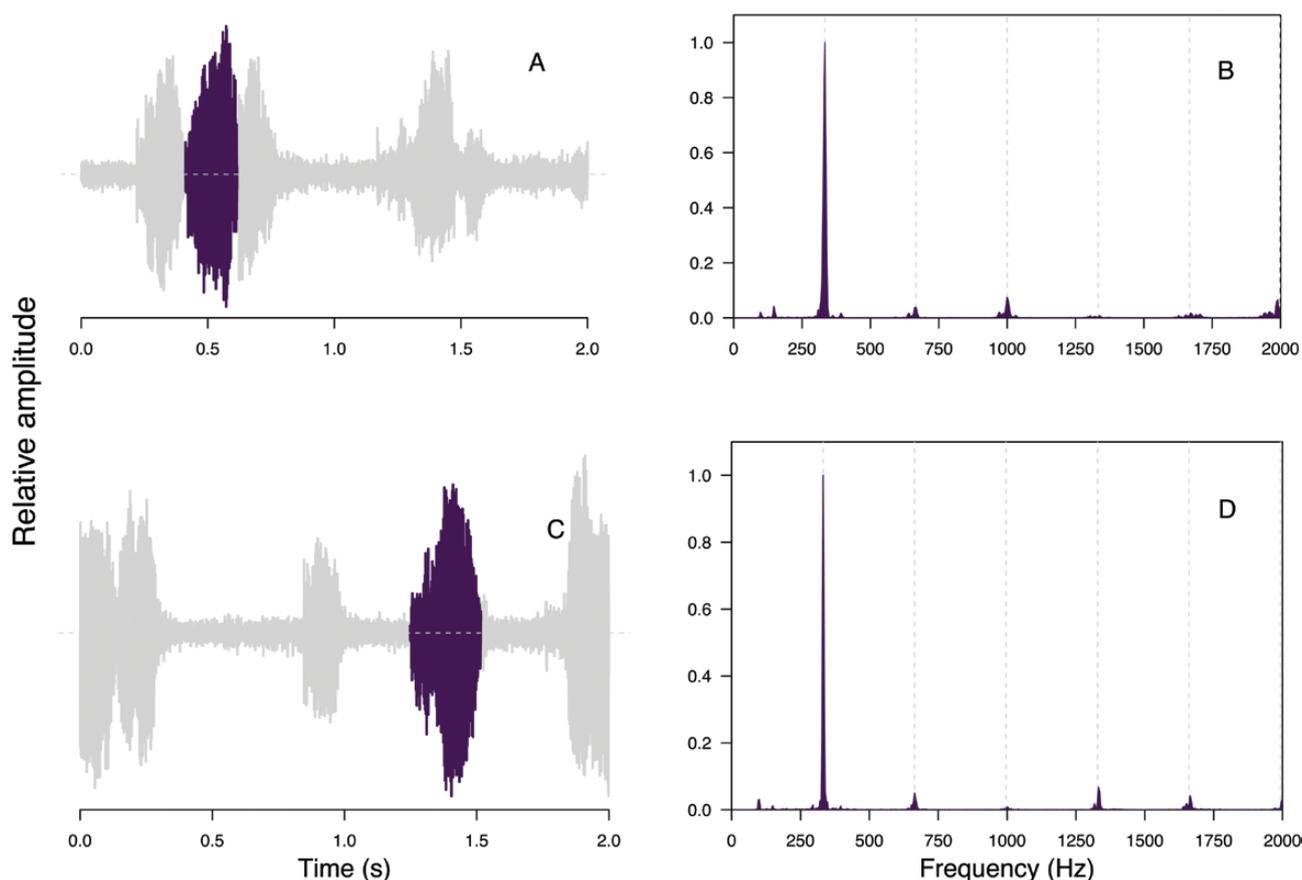


Figure 5. Time and frequency characteristics of floral vibrations produced by the same individual of *Bombus terrestris* *audax* on buzz-pollinated flowers of *Solanum citrullifolium* (A, B) or *S. heterodoxum* (C, D) registered with an audio recorder placed at 10 cm from the flower. Time domain: panels A and C show multiple buzzes (floral vibrations) produced over 2 s of a floral visit. Frequency domain: panels B and D show the power spectral density (PSD) of the floral vibration highlighted in purple in the corresponding oscillograms in A and C. The highest peak in the PSD corresponds to the fundamental frequency (333 Hz for *S. citrullifolium* and 332 Hz for *S. heterodoxum*). The first five harmonics of the fundamental frequency are shown with vertical dashed lines (B, D). For plotting, we applied a bandpass filter (50–5000 Hz), and estimated the PSD using a Hamming window (length = 2048 samples).

Table 3. Effect of plant species and bout number on the fundamental frequency (Hz) of floral vibrations produced by *Bombus terrestris* visiting flowers of two *Solanum* taxa. Model estimates and *P*-values were obtained using type III sums of squares of the fixed effects of a linear mixed-effects model. SE = standard error

Model component	Estimate	SE	<i>P</i> -value
Intercept	348.734	3.397	
Plant species (<i>S. heterodoxum</i>)	-4.502	0.966	0.008
Bout number	-0.733	0.150	0.002

et al., 2019; Rosi-Denadai *et al.*, 2020). In contrast, the mean natural frequency of the pollinating anthers of the large-flowered taxa (*S. rostratum*, *S. grayi* var.

grandiflorum and *S. citrullifolium*) falls below the bee range of 100–400 Hz. The functional consequence of this dissonance in fundamental frequencies among stamen types is unknown. It is possible that some bees may be able to induce stamen resonance by matching their floral vibrations to the natural frequency of the feeding stamens, but the same frequency will not induce resonance in pollinating stamens, potentially controlling pollen dispensing (Dellinger *et al.*, 2019a; Kemp & Vallejo-Marín, 2020).

For the small-flowered taxa (*S. fructu-tecto*, *S. grayi* var. *grayi* and *S. heterodoxum*), the mean difference in natural frequency among stamen types is smaller, and their ranges largely overlap. This suggests that the evolutionary transition from large to small flowers in this group (Vallejo-Marín *et al.*, 2014) is associated with the convergence of vibrational properties between stamen types. Further studies

with increased replication in heterantherous taxa with shifts in the expression of anther dimorphism, or during evolutionary transitions between buzz pollination and other modes of pollination (Brito *et al.*, 2016; Dellinger *et al.*, 2019b), might allow one to test the hypothesis that evolutionary shifts in stamen function are accompanied by changes in vibrational properties.

The effect of stamen type on natural frequency occurs in addition to differences in length associated with the two anther types (which were accounted for in our statistical models), suggesting that other stamen characteristics influence the vibrational properties of different types of anthers. Finer characterization of stamen morphological properties (e.g. through analysis of X-ray, micro-computed tomography scanning, as in Dellinger *et al.*, 2019c) and their material properties (Mortimer, 2017; Saltin *et al.*, 2019) might help in elucidating the mechanism by which the vibrational properties of these anther types are determined. Because stamens are relatively complex structures and not simple cantilever beams, modelling approaches such as finite element modelling (FEM) (Saltin *et al.*, 2019) could seek to integrate these morphological and material properties to generate predictions of the relationship between floral traits and vibrational properties. Moreover, variation within species (e.g. between varieties of *S. grayi*) also opens opportunities to increase the segregating variation within experimental populations through artificial crosses (Conner, 2003) and disentangle the contribution of correlated floral traits to variation in natural frequencies.

NATURAL FREQUENCY OF INDIVIDUAL STAMENS AND POLLEN RELEASE

The overlap between the range of frequencies produced by some bees and those of the studied *Solanum* flowers suggests that, in certain taxa, resonance might come into play during buzz pollination, potentially increasing the magnitude of the vibrations applied by bees and, hence, increasing pollen release. A caveat with using our results to interpret the effect of resonance during buzz pollination is that the response of a stamen to vibrations at its natural frequency is likely to change as the bee firmly holds the anther with its mandible and presses its body against these floral parts (Buchmann, 1983). Future studies will benefit from exploring the resonance of coupled bee-flowers, although the technical challenges to acquire these data are significant. At the least, the natural frequencies of free stamens we calculated provide insight into the potential for resonance to increase pollen release in some types of flowers, including heterantherous flowers such as those studied here. In many heterantherous

flowers, pollinators usually manipulate a subset of the anthers in the flower (feeding anthers) during visitation (Luo, Zhang & Renner, 2008; Vallejo-Marín *et al.*, 2009). In some cases, a set of anthers (usually the pollinating anthers) remains free during floral vibrations (Vallejo-Marín *et al.*, 2009). Furthermore, relatively small buzz-pollinators interact with only some of the stamens even in non-heterantherous flowers (Solís-Montero *et al.* 2015). If the floral visitors vibrate the flower at the natural frequency of the free stamens measured here, then, in principle, resonance at these frequencies could increase pollen delivery during visitation.

DOES STAMEN RESONANCE PLAY A ROLE IN POLLEN RELEASE DURING BUZZ POLLINATION?

If stamen resonance at the natural frequencies is within the reach of at least some bee species, the question arises as to whether bees exploit this resonance effect during buzz pollination. Empirical work with bumblebees suggests that this is not the case. Our bumblebee experiment suggests that *B. terrestris* do not rapidly match their floral buzzes to the natural frequency of the flowers they visit. Our results are consistent with previous work on bumblebees that also showed a lack of frequency adjustment to match specific vibrations in bees visiting different types of mechanical flowers that released pollen when buzzed at specific frequencies (Switzer *et al.*, 2019).

The lack of dynamic adjustment between bee floral buzzes and the natural frequency of stamens over consecutive visits of an individual bee to the same flower could be explained if an individual bee is unable to change the frequency of the vibrations produced during floral visitation. Previous work has shown that the frequency of floral buzzes decreases with experience at manipulating buzz-pollinated flowers, while simultaneously resulting in more pollen being collected per visitation bout (Whitehorn, Wallace & Vallejo-Marín, 2017). In this case, the change in the fundamental frequency of floral buzzes is relatively small [~ 20 Hz over ten visitation bouts in Whitehorn *et al.* (2017), and ~ 1 – 20 Hz over six visitation bouts in the present study]. Thus, it is possible that there are narrow limits to the adjustment in frequency that a bee can achieve during buzz pollination. However, empirical work has shown that bumblebees can significantly change their buzz frequencies in other types of non-flight vibrations. For example, the defence buzzes produced by *B. terrestris* have a significantly lower frequency (236.32 ± 4.29 Hz) than those produced on flowers (313.09 ± 2.63 Hz) (Pritchard & Vallejo-Marín, 2020). This suggests that changes in the frequency of non-flight vibrations of larger magnitude (~ 80 Hz) compared to those we

observed (~20 Hz) are at least possible in the same individual bee. The behavioural mechanism that would allow a bumblebee to associate the production of a particular buzz frequency with an increase in pollen release is unclear. Perhaps inexperienced bees initially produce buzzes of variable frequencies and over time learn to associate particular frequencies with increased pollen release (due to resonance), but the elegant experiment of Switzer *et al.* (2019) with mechanical flowers provided no evidence of this type of instrumental learning.

Alternatively, producing vibrations at the resonant frequency of stamens might have a relatively small effect on pollen release compared to the effect of other components of the bee's buzz such as amplitude, and the duration and number of buzzes (King & Buchmann, 1996; De Luca *et al.*, 2013). Experiments applying bee-like vibrations of different characteristics show that pollen release is more strongly determined by vibration amplitude (peak velocity) than by frequency in *S. rostratum* (De Luca *et al.*, 2013). The lower frequency vibrations produced by *B. terrestris* during defence are also lower in amplitude compared to the higher frequency, higher acceleration amplitude buzzes producing during floral visitation (Pritchard & Vallejo-Marín, 2020). Vibrations of both low frequency and high acceleration may not be possible to reach by bees if their maximum displacement is reached, e.g. due to the limits imposed by thoracic size (Corbet & Huang, 2014). If the gain in increased pollen release that would be achieved through stamen resonance is offset by a decrease in pollen release due to producing vibrations of lower amplitude, then bees might not benefit from matching the relatively lower natural frequency of flowers. Instead, the optimal frequency of the floral vibrations by a bee to maximize pollen release may be best explained by the resonance properties of the body of the bee. Buzz-pollinating bees may benefit from vibrating at the resonance frequency of their own bodies (King, 1993; King & Buchmann, 2003), which would produce the highest amplitude vibration for a given input of energy.

CONCLUSIONS

For large bees that can reach amplitudes high enough to elicit pollen release, stamen natural frequency may not be the most important determinant of the type of vibrations they produce. However, exploiting the vibrational properties of stamens may be advantageous for other bees unable to reach the required acceleration amplitudes to elicit pollen release due to small size, low mass or other

biomechanical constraints (King & Buchmann, 2003). For these smaller bees, the increase in vibration amplitude potentially achieved through resonance of stamens might allow them to utilize flowers that would otherwise be beyond their vibrational reach. Inducing resonance of poricidal stamens may also be useful as a mechanism to increase pollen release in species of agricultural importance such as tomato (*S. lycopersicum* L.), eggplant (*S. melongena* L.) and kiwifruit [*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson], for which mechanical shakers are sometimes used to pollinate crops. Further work should compare the stamen natural frequency of other buzz-pollinated flowers with buzzing frequencies of a broader community of visiting bees to establish whether any bee exploits floral resonance for pollen release. By building on classical work on the biomechanics of buzz pollination (e.g. King, 1993; King & Buchmann, 1996) our work suggests new and exciting lines of inquiry integrating biomechanics and ecological interactions at the organismal level (Bauer, Poppinga & Müller, 2020).

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DATA ACCESSIBILITY

Data and code are publicly available at <http://hdl.handle.net/11667/153>.

AUTHOR CONTRIBUTIONS

C.E.P.N. carried out plant experiments, analysed the data, participated in study design and drafted the manuscript. L.N. carried out bee experiments, analysed the data, participated in study design and commented on the manuscript. F.M.Z. participated in the experimental design and commented on the manuscript. M.V.M. participated in conceiving the study and data analysis and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Information on the origin of seeds of the six taxa of *Solanum* section *Androceras* studied here. n = number of flowers analysed.

Table S2. Summary statistics of 13 floral traits of the morphology and mass measured in six heterantherous taxa of *Solanum* section *Androceras*. n = number of flowers analysed (54 flowers in total). FS = feeding stamen; PS = pollinating stamen; CRW = corolla width; CRH = corolla height; FLM = flower mass; STL = stamen length; ANL = anther length; FIL = filament length; ADL = anther major diameter; ADS = anther minor diameter; FDL = filament major diameter; FDS = filament minor diameter; STM = stamen mass; ANM = anther mass; FIM = filament mass.

Figure S1. Numerical matrix depicting the Pearson product-moment correlations among 13 floral and stamen traits and the natural frequency of stamens across six taxa of heterantherous, buzz-pollinated species of *Solanum* section *Androceras*. Correlations were calculated separately for pollinating stamens (upper triangle of the matrix) and feeding stamens (lower triangle). Negative correlations are shown in red and positive correlations in blue, with darker colours indicating higher absolute values. The order of the variables shown in the figure were chosen using hierarchical clustering.