

Buzz pollination in eight bumblebee-pollinated *Pedicularis* species: does it involve vibration-induced triboelectric charging of pollen grains?

Sarah A. Corbet¹ and Shuang-Quan Huang^{2,*}

¹*St Loy Cottages, St Buryan, Penzance, Cornwall TR19 6DH, UK and* ²*School of Life Sciences, Central China Normal University, Wuhan 430079, China*

* For correspondence. E-mail hsq@mail.ccnu.edu.cn

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• **Background and Aims** Buzz pollination involves explosive pollen release in response to vibration, usually by bees. The mechanism of pollen release is poorly understood, and it is not clear which component of vibration (acceleration, frequency, displacement or velocity) is critical; the role of buzz frequency has been particularly controversial. This study proposes a novel hypothesis that explosive pollen release results from vibration-induced triboelectric charging. If it does, pollen release is expected to depend on achievement of a critical threshold velocity.

• **Methods** Eight sympatric buzz-pollinated species of *Pedicularis* that share bumblebee pollinator species were studied, giving a rare opportunity to compare sonication behaviour of a shared pollinator on different plant species.

• **Key Results** Reconsidering previous experimental studies, it is argued that they establish the critical role of the velocity component of vibration in pollen release, and that when displacement is constrained by body size bees can achieve the critical velocity by adjusting frequency. It was shown that workers of *Bombus friseanus* assorted themselves among *Pedicularis* species by body size, and that bees adjusted their buzz/wingbeat frequency ratio, which is taken as an index of the velocity component, to a value that corresponds with the galea length and pollen grain volume of each species of *Pedicularis*.

• **Conclusions** Sonication behaviour of *B. friseanus* differs among *Pedicularis* species, not only because worker bees assort themselves among plant species by body size, but also because bees of a given size adjust the buzz frequency to achieve a vibration velocity corresponding to the floral traits of each plant species. These findings, and the floral traits that characterize these and other buzz-pollinated species, are compatible with the hypothesis of vibration-induced triboelectric charging of pollen grains.

Key words: *Bombus*, bumblebee pollinators, buzz frequency, buzz pollination, electrostatics, floral traits, *Pedicularis*, pollination, sonication behaviour, sympatric species, triboelectric charge.

INTRODUCTION

Buzz pollination involves sudden release of a puff of pollen in response to vibration (sonication, buzzing) by a visiting insect (nearly always a bee). Many buzz-pollinated flowers share striking features, such as elongate poricidal anthers (Buchmann, 1983), the functional significance of which is not obvious. The mechanism of buzz pollination remains elusive and the relative roles of the components of vibration – frequency, velocity, displacement and acceleration – are unclear (King and Buchmann, 1996). The role of buzz (vibration) frequency, the only component that can be measured directly in the field without sophisticated equipment, is particularly controversial; it has been claimed that variation in frequency has no significant effect on pollen release by sonication (Harder and Barclay, 1994; de Luca and Vallejo-Marin, 2013; de Luca *et al.*, 2013). We revisit that claim.

Pollen grains can become electrostatically charged and can move in an electrostatic field (Corbet *et al.*, 1982; Bannerjee and Law, 1988; Gan-Mor *et al.*, 1995, 2009; Vaknin *et al.*, 2000; Bechar *et al.*, 2008), and this property is used commercially in the electrostatic charging of pollen for pollination of kiwifruit and other crops (Gan-Mor *et al.*, 1995, 2009;

Bechar *et al.*, 2008). Electrostatic charging has been implicated in buzz pollination (Buchmann and Hurley, 1978; Buchmann, 1983; Erickson and Buchmann, 1983). Here we propose a hypothesis that the explosive emission of pollen on sonication is due to vibration-induced triboelectric charging of pollen grains within the anther (or, in *Pedicularis*, within the galea). Because triboelectric charging depends on the velocity component of vibration (Matsusaka *et al.*, 2010), we explore the possible role of this component by reconsidering earlier published experimental studies and by a comparative field study of the relationship between sonication behaviour and floral traits in eight sympatric buzz-pollinated congeneric species that share bumblebee pollinator species.

Many species of *Pedicularis* are buzz-pollinated by bumblebees (Macior *et al.*, 2001). In China there are more than 350 species of *Pedicularis* (Yang *et al.*, 1998). Taking advantage of the abundance and diversity of species of *Bombus* and *Pedicularis* in the Hengduan Mountain region in Yunnan, south-west China, we explored the relationship between buzzing behaviour and the floral traits of different species of *Pedicularis*, and we ask whether the findings of this and earlier studies are compatible with our hypothesis that pollen activation by sonication results from triboelectric charging of pollen grains.

MATERIALS AND METHODS

The flowers

There is great diversity in the form of the flowers of *Pedicularis* (Fig. 1; Li, 1951; Yang *et al.*, 1998). The upper lip forms a galea, which in many species is modified to enclose the anthers, and is extended into a long, slender beak. The beak is asymmetrically positioned in the flower, and its length and curvature vary among species (Macior, 1968; Ree, 2005; Huang and Fenster, 2007; Eaton *et al.*, 2012). The beak is curved, sometimes (as in *P. integrifolia*) dramatically so (Fig. 1). Its shape dictates the position of pollen placement and stigma contact on a visiting bee (Huang and Shi, 2013). The anthers of *Pedicularis* are not poricidal, but in beaked species they are enclosed within the cavity of the galea. Huang and Shi (2013) showed that pollen issues vigorously from the tip of the beak when a bee vibrates the galea, and that sympatric species of *Pedicularis* differ with respect to the position adopted by bees buzzing for pollen, and the corresponding position of pollen placement and stigma contact on the bee's body.

Eight *Pedicularis* species were studied in uncultivated alpine meadows at the Shangri-La Alpine Botanic Garden (27°54'5"N, 99°38'17"E, 3300–3500 m a.s.l.) in Yunnan Province, south-west China, in July and August 2012 and 2013, comprising representatives of the three major types of floral form recognized by Li (1951) and Ree (2005): nectar-producing, short-tubed, non-beaked species (*P. densispica* and *P. rex*), largely nectarless,

short-tubed, beaked species (*P. oxycarpa* and *P. dichotoma*) and nectarless, long-tubed, beaked species (*P. tricolor*, *P. longiflora*, *P. siphonantha* and *P. cephalantha*). We measured floral traits on 20 specimens of each of the eight species of *Pedicularis* (corolla tube length, lower lip width, and galea length measured in a straight line from base to tip ('galea straight' lengths, $n = 50$ of each species). Galea lengths measured along the curve ('galea curve' lengths) are taken from Yang *et al.* (1998). The diameter of pollen grains fixed in FAA were measured from 20 plants of each species from the same site (Tang and Huang, 2007). The mean trait values for each species of *Pedicularis* (Table 1) were used in the analyses. Plant names follow Yang *et al.* (1998).

The bees

In deference to local Buddhist sentiment the project was designed to avoid killing bees. It is not feasible to measure acceleration, velocity or displacement in the field without special equipment, but buzz frequency and wingbeat frequency in flight (henceforth 'wingbeat frequency') can be measured by acoustic recording. If smaller bumblebees have a higher wingbeat frequency than large individuals of the same species at a given temperature, wingbeat frequency in flight might be a surrogate for body size. Individual bees [workers of *Bombus friseanus* Skorikov (previously referred to as *B. richardsi*) and *Bombus festivus* Smith] were followed as they foraged for pollen on

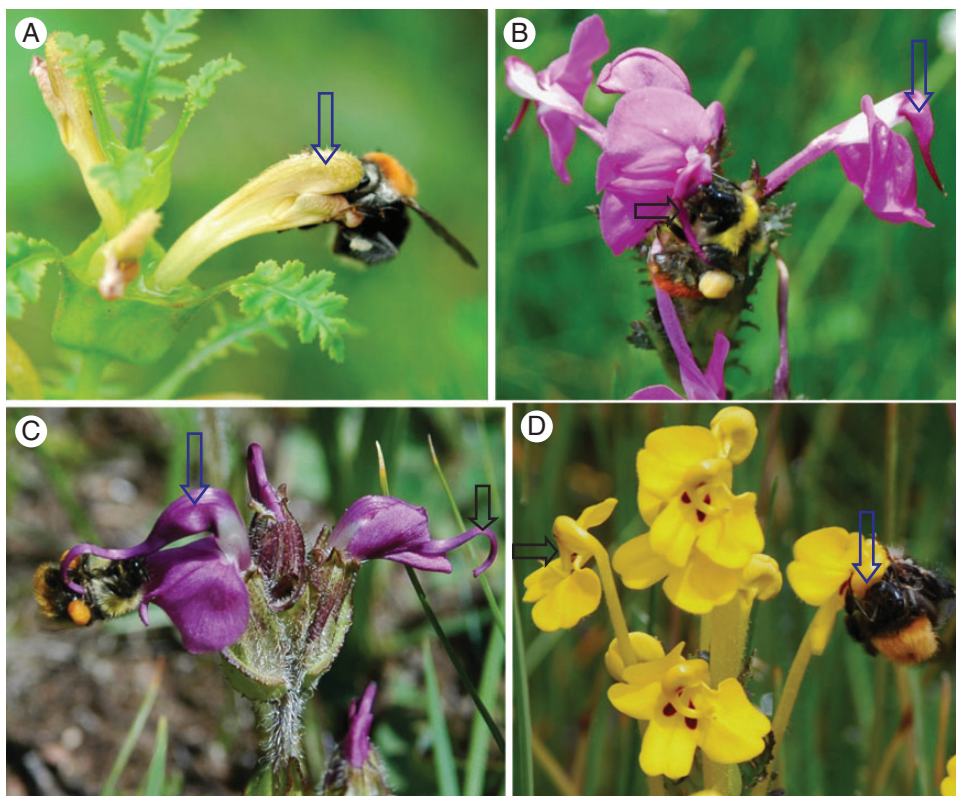


FIG. 1. Flowers of four species of *Pedicularis*, with sonicating bumblebees. (A) A beakless, nectarless species, *P. rex*, with *B. festivus* (note pollen grains on the bee's head and left dorsal thorax). (B) A beaked species, *P. cephalantha*, with *B. friseanus*. (C) A beaked species, *P. integrifolia*, showing the strongly curved beak, with *B. friseanus*. (D) A beaked, long-tubed species, *P. longiflora*, with *B. festivus*. Black arrows mark the beak and blue arrows show the position of the anthers within the galea.

TABLE 1. Floral traits for a flower from each of 20 specimens (50 for galea straight, measured in a direct line from the base to the tip) of each of eight species of *Pedicularis*. Galea curve was measured along the curve. Data are means (s.e.m.)

Pedicularis species	Corolla tube (mm)	Galea straight (mm)	Galea curve (mm)	Lower lip width (mm)	Pollen grain volume (μm^3)
<i>P. longiflora</i>	53.26 (1.201)	4.212 (0.306)	6	22.20 (0.213)	21 187 (507.11)
<i>P. cephalantha</i>	19.10 (0.308)	8.503 (0.696)	5.5	23.94 (0.259)	10 407 (232.97)
<i>P. siphonantha</i>	57.56 (1.875)	3.848 (0.498)	11	20.4 (0.259)	13 575 (362.21)
<i>P. oxycarpa</i>	7.118 (0.119)	6.835 (0.585)	4	14.01 (0.235)	7345 (118.38)
<i>P. rex</i>	23.43 (0.498)			12.71 (0.382)	4448 (89.28)
<i>P. densispica</i>	7.28 (0.195)			10.63 (0.223)	2169 (40.50)
<i>P. tricolor</i>	57.32 (1.635)	5.682 (0.287)	16	35.78 (0.491)	15 074 (209.41)
<i>P. dichotoma</i>	14.93 (1.21)	7.397 (0.488)	10	13.91 (0.215)	5586 (55.63)

Pedicularis species, and their buzzes and flight tones were recorded digitally. The recordings were analysed using RavenLite sound analysis software (Charif *et al.*, 2006). After extending the recorded waveform on the time axis, the pitch of each buzz and the flight tone were estimated by counting the major cycles in one-tenth of a second (Fig. 2). The analyses are based on the means for individual bees (sequences of 1–28 buzzes and 1–22 flights for each bee), or on overall means, i.e. the means of these individual mean values for bees visiting each of the eight species of *Pedicularis*. The numbers of buzz sequences recorded for *B. friseanus* and *B. festinus* were 6 and 29 respectively on *P. cephalantha*, 5 and 0 on *P. densispica*, 8 and 0 on *P. dichotoma*, 3 and 4 on *P. longiflora*, 4 and 1 on *P. oxycarpa*, 1 and 0 on *P. rex*, 3 and 1 on *P. siphonantha* and 10 and 0 on *P. tricolor*; in this study *B. festinus* was not seen buzzing *P. densispica*, *P. dichotoma*, *P. rex* or *P. tricolor* (Table 2).

A sonicating bee emits pulses of vibration. The number and duration of pulses of buzzing between two episodes of flight were estimated from the extended waveform plot, omitting any potentially incomplete sequences of buzzes at the start of a recording, using data from 2012. The numbers of these sequences for *B. friseanus* and *B. festinus* were 5 and 8 respectively on *P. cephalantha*, 5 and 0 on *P. densispica*, 3 and 4 on *P. longiflora*, 3 and 1 on *P. oxycarpa*, 3 and 0 on *P. siphonantha* and 7 and 0 on *P. tricolor*.

Some bees were caught for weighing or measurement. Bees were chilled, squeezed gently to force regurgitation of their nectar, deprived of their pollen loads and weighed on a Sartorius microbalance precise to 0.0001 g. Thorax widths were measured in the field with digital callipers on individuals held between a sponge-rubber piston and a delicate polythene membrane (Clingfilm, Saranwrap) at the end of a honeybee queen-marking tube. We used the mean of ten measurements for each bee. All bees were then released.

Bee names follow Williams *et al.* (2009). Statistical analyses were performed in R (R Development Core Team, 2012).

RESULTS

For *B. friseanus*, regression analysis showed a significant relationship between wingbeat frequency and ln body weight ($R^2 = 0.65$, $F_{1,19} = 38.94$, $P < 0.001$) (Fig. 3A) and between wingbeat frequency and thorax width ($R^2 = 0.41$, $F_{1,8} = 7.73$, $P = 0.03$) (Fig. 3B). We therefore use wingbeat frequency as an index of body size.

Using means for individual bees, buzz frequency was significantly dependent on the wingbeat frequency of a bee flying between flowers on which it was buzzing, for all bees or for *B. friseanus* considered alone ($P \leq 0.001$, Table 3A, E). For *B. friseanus* alone, analysis based on overall means showed that the buzz frequency increased with wingbeat frequency (Fig. 4A, Table 4). Workers of *B. festinus* showed less variation in body size than those of *B. friseanus*, and we have data for their visits to only four *Pedicularis* species. Our sparse data indicated that, in this species also, buzz frequency depended on wingbeat frequency ($P = 0.008$) (Table 3G). Bees with a higher wingbeat frequency (i.e. smaller bees) had a higher buzz frequency.

Wingbeat frequency varied with bee species ($P < 0.0001$) and flower species ($P < 0.0001$), with a marginally significant interaction between bee species and flower species ($P = 0.05$) (Table 3A). The relationship between wingbeat frequency and flower species is unlikely to be due to a direct effect of flower species on wingbeat frequency; it is more likely that individual buzzing worker bees partitioned themselves among flower species by body size (reflected in wingbeat frequency).

Evidently differences in buzz frequency between *Pedicularis* species were due at least in part to the size-based partitioning of worker bees among flower species. The ratio of buzz frequency to wingbeat frequency is taken to represent the velocity component of vibration (see Discussion). For all bees pooled, this ratio varied with wingbeat frequency ($P < 0.0001$) and bee species ($P = 0.009$) (Table 3B).

Collinearity among floral traits complicates the interpretation of relationships between bee behaviour and floral traits. For example, among our eight species of *Pedicularis* mean pollen grain volume correlated with corolla tube length and lower lip width (Table 4) and style length (Yang and Guo, 2004), and corolla tube length correlated with galea straight length (Table 4).

Because we have more data for *B. friseanus*, and because of its greater size variation, correlations with floral traits are based on this species alone. Multiple regression, using the mean values of wingbeat frequency, buzz frequency and buzz/wingbeat frequency ratio for individual *B. friseanus* workers and species means for floral traits, showed that wingbeat frequency was significantly related to the width of the lower lip ($P < 0.001$) (Table 5A, Fig. 4B). Buzz frequency was associated with wingbeat frequency ($P < 0.001$) and galea curve length ($P = 0.02$) (Table 5B), but not galea straight length (Fig. 4D). The buzz/wingbeat frequency ratio was associated with wingbeat frequency ($P = 0.009$) and galea straight length ($P = 0.02$) (Table 5C).

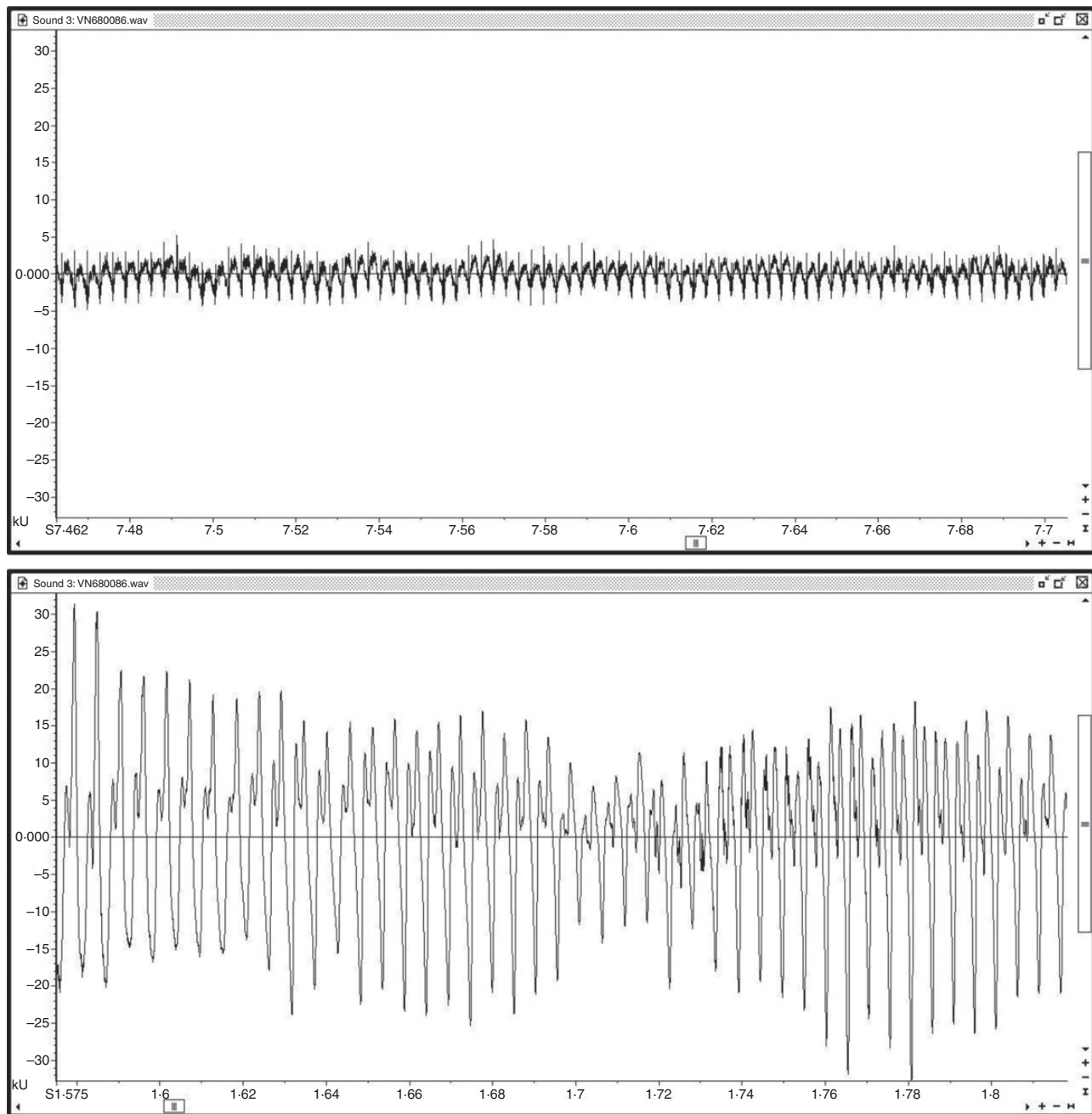


FIG. 2. An example of RavenLite output: waveform of a buzz (above) and flight (below) for *B. friseanus* on *P. tricolor*. Time base in seconds.

Based on overall means for *B. friseanus*, Fig. 4 illustrates the relationship in beaked species between wingbeat frequency and lower lip width (larger bees choosing *Pedicularis* species with a wider lower lip) (Fig. 4B), and shows that in beaked species the buzz frequency decreased with increase in galea curve length (Fig. 4C), and that the buzz/wingbeat frequency ratio was positively related to galea straight length (Fig. 4E) and inversely related to pollen grain volume (log-transformed) (Fig. 4F).

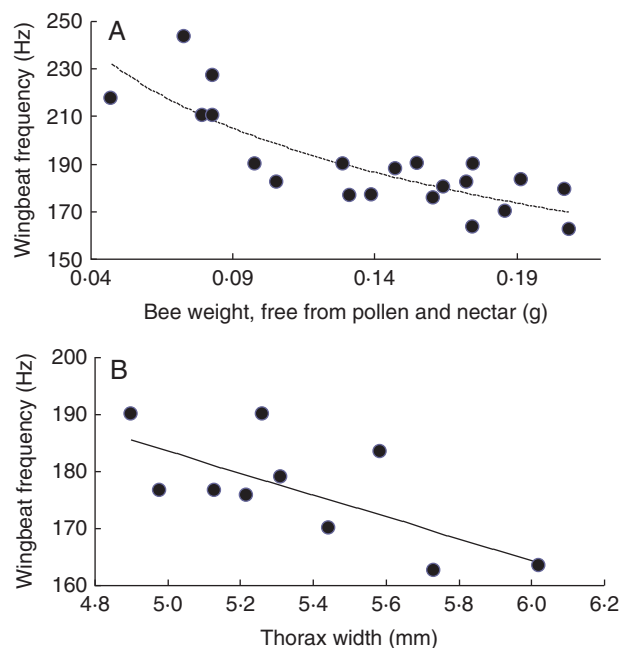
We conclude that workers of *B. friseanus* assorted themselves by body size among species of *Pedicularis* and that, after

wingbeat frequency (body size) had been taken into account, the buzz frequency varied with plant species in a manner that depended on galea curve length (or correlates) and the buzz/wingbeat frequency ratio varied with galea straight length and pollen grain volume (Fig. 4E, F).

Does the temporal patterning of pulses of buzzing vary with species of *Pedicularis* or species of bee? When bees of both species were pooled, the mean number of pulses per flower visit showed a significant effect of bee species ($P = 0.01$) (Tables 6 and 7A), being greater in *B. festivus* than in *B. friseanus*, and the mean duration of each pulse showed

TABLE 2. Wingbeat frequency, buzz frequency and buzz/wingbeat frequency ratio of *B. friseanus* and *B. festivus* on eight species of *Pedicularis*. Data are means (s.e.m.); each mean is the overall mean of the means for *n* individual bees

	<i>Pedicularis</i> species							
	<i>cephalantha</i>	<i>densispica</i>	<i>dichotoma</i>	<i>longiflora</i>	<i>oxycarpa</i>	<i>rex</i>	<i>siphonantha</i>	<i>tricolor</i>
<i>B. friseanus</i>								
Wingbeat frequency, Hz	207.6 (11.16) <i>n</i> = 6	187.8 (8.72) <i>n</i> = 5	224.7 (5.29) <i>n</i> = 8	234.6 (8.47) <i>n</i> = 3	229.9 (4.27) <i>n</i> = 4	180 <i>n</i> = 1	215.5 (11.28) <i>n</i> = 3	189.2 (7.02) <i>n</i> = 10
Buzz frequency, Hz	311.1 (5.71) <i>n</i> = 6	302.9 (17.81) <i>n</i> = 5	308.1 (7.04) <i>n</i> = 8	318.8 (17.82) <i>n</i> = 3	342.5 (10.66) <i>n</i> = 4	276.7 <i>n</i> = 1	290.7 (2.99) <i>n</i> = 3	271.3 (15.44) <i>n</i> = 10
Buzz/wingbeat frequency ratio	1.524 (0.097) <i>n</i> = 6	1.613 (0.052) <i>n</i> = 5	1.377 (0.046) <i>n</i> = 8	1.357 (0.031) <i>n</i> = 3	1.493 (0.072) <i>n</i> = 4	1.537 <i>n</i> = 1	1.356 (0.069) <i>n</i> = 3	1.434 (0.066) <i>n</i> = 10
<i>B. festivus</i>								
Wingbeat frequency, Hz	188.81 (6.428) <i>n</i> = 29			186.45 (11.123) <i>n</i> = 4	193.3 <i>n</i> = 1		130 <i>n</i> = 1	
Buzz frequency, Hz	276.27 (5.434) <i>n</i> = 29			266.68 (15.94) <i>n</i> = 4	322.5 <i>n</i> = 1		290 <i>n</i> = 1	
Buzz/wingbeat frequency ratio	1.617 (0.047) <i>n</i> = 29			1.639 (0.058) <i>n</i> = 4	1.506 <i>n</i> = 1		2.231 <i>n</i> = 1	

FIG. 3. Relationship between wingbeat frequency and (A) body weight and (B) mean thorax width for *B. friseanus*.

significant effects of flower species ($P = 0.01$) (Table 7C). When *B. friseanus* was considered alone, the mean duration of each pulse showed a significant effect of flower species ($P = 0.05$) (Table 7E). The total buzzing time per flower showed a weak effect of flower species (Table 7D). The effect of plant species on pulse duration and total buzzing time per flower (Table 7D, E) was due to *B. friseanus* giving very brief pulses on *P. densispica* (which has no beak); the effect of plant

species on pulse duration and total buzzing time was no longer significant when *P. densispica* was omitted ($P = 0.35$ and $P = 0.52$ respectively for the five beaked species).

Examination of an oiled slide held under a buzzing *B. friseanus* on *P. dichotoma* showed that the pollen was released in small clumps.

DISCUSSION

Determinants of wingbeat frequency and buzz frequency in different *Pedicularis* species

Our analyses indicated that in *B. friseanus* wingbeat frequency correlated with size, as measured by thorax width or body weight (Fig. 3). Worker bees were assorting themselves among *Pedicularis* species by body size. Macior (1982) found that *Pedicularis* species sharing pollinator species partitioned their pollinators by caste (effectively by size), and Peat *et al.* (2005) demonstrated size-based partitioning within the worker caste in nectar-foraging *Bombus terrestris*. We have shown size-based partitioning within the worker caste among pollen-foraging individuals of *B. friseanus*. This partitioning was related to lower lip width (Table 5A); larger individual bees visited larger-flowered species of *Pedicularis* (Fig. 4B).

Buzz frequency was significantly, but not solely, dependent on wingbeat frequency. The buzz/wingbeat frequency ratio was remarkably highly correlated with galea straight length and pollen grain volume (Fig. 4E, F). These findings suggest that a bee of a given size could adjust its buzz frequency in relation to these or associated floral traits.

Pollen activation by sonication

It is helpful to distinguish two types of activation of pollen grains by sonication: explosive release and 'boiling' (King and

TABLE 3. Analyses of variance (A, B, D, F) and covariance (C, E, G) for the relationship between wingbeat frequency, buzz frequency and buzz/wingbeat frequency ratio with bee species and flower species, for all bees (A–D) and for *B. friseanus* (E, F) and *B. festivus* (G) separately. Interactions with $P > 0.1$ are not shown. In *B. festivus* buzz frequency was significantly related to wingbeat frequency, but wingbeat frequency showed no significant effect of flower species

	Dependent variable	Independent variable	d.f.	Mean square	F	P
A	Buzz frequency, all bees	Bee species	1	10 930	11.53	0.001
		Flower species	7	19 228	2.893	0.01
B	Wingbeat frequency, all bees	Bee species	1	60 399	209.36	<0.0001
		Flower species	7	1497	5.199	<0.0001
		Bee × flower	3	786	2.725	0.049
C	Buzz frequency, all bees	Wingbeat frequency	1	28 462	36.76	<0.0001
D	Buzz/wingbeat frequency ratio, all bees	Bee species	1	2.211	71.973	<0.0001
E	Buzz frequency, <i>B. friseanus</i>	Wingbeat frequency	1	15 678	20.987	<0.0001
		Wingbeat × flower	6	1646.9	2.205	0.074
F	Wingbeat frequency, <i>B. friseanus</i>	Flower species	7	1841.9	4.765	<0.001
G	Buzz frequency, <i>B. festivus</i>	Wingbeat frequency	1	6070	8.046	0.008

Bold: $P < 0.05$.

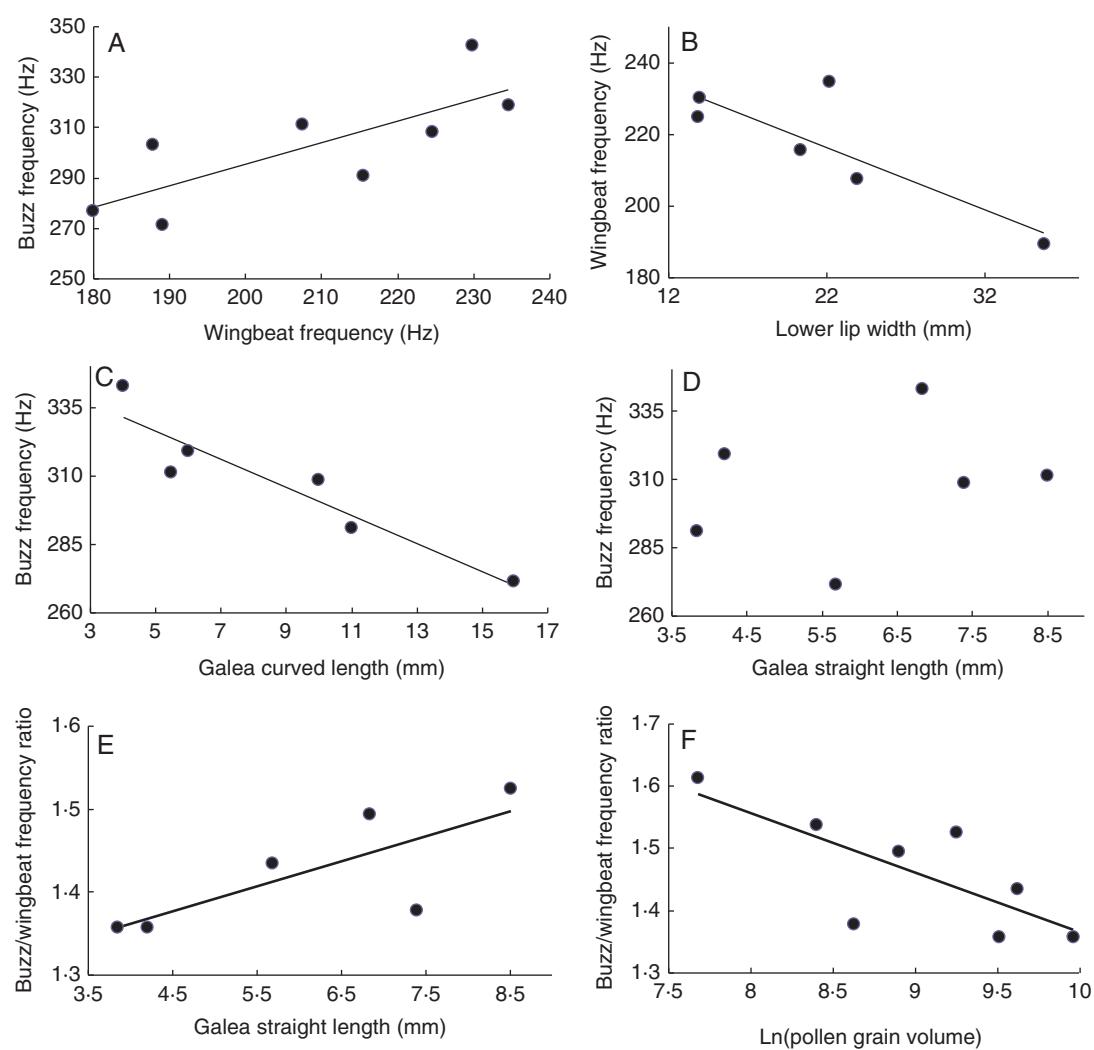


FIG. 4. *Bombus friseanus*: relationship between overall mean values for each *Pedicularis* species of (A) buzz frequency and wingbeat frequency, (B) wingbeat frequency and lower lip width, (C) buzz frequency and galea curve length, (D) buzz frequency and galea straight length, (E) buzz/wingbeat frequency ratio and galea straight length and (F) buzz/wingbeat frequency ratio and ln pollen grain volume (μm^3). Only beaked species are included in B, C, D and E. Regression lines are shown where the relationship is significant at $P < 0.05$; buzz/wing beat frequency ratio was significantly related to galea straight length, but buzz frequency was not.

TABLE 4. Pearson's product-moment correlation coefficients (and probability) for sonication behaviour of *B. friseanus* and floral traits, based on the means for each of the eight species of *Pedicularis*. Correlations involving galea lengths and lower lip width are based on the beaked species only, omitting *P. densispica* and *P. rex*

	Buzz frequency	Buzz/wingbeat ratio	Lower lip width	Corolla tube length	Galea curve	Galea straight	Ln (pollen volume)
Wingbeat frequency	0.771 (0.03)	-0.643 (0.09)	-0.831 (0.04)	0.064 (0.88)	-0.734 (0.097)	-0.141 (0.79)	0.483 (0.22)
Buzz frequency		-0.010 (0.98)	-0.763 (0.08)	-0.479 (0.23)	-0.935 (0.006)	0.299 (0.56)	0.011 (0.98)
Buzz/wingbeat ratio			0.086 (0.87)	-0.684 (0.06)	-0.377 (0.46)	0.762 (0.08)	-0.750 (0.03)
Lower lip width				0.632 (0.09)	0.651 (0.16)	-0.193 (0.71)	0.735 (0.04)
Corolla tube length					0.611 (0.20)	-0.831 (0.04)	0.800 (0.02)
Galea curve						-0.307 (0.55)	0.202 (0.70)
Galea straight							-0.702 (0.12)

Bold: $P < 0.05$.

TABLE 5. Multiple regression analysis of floral traits (curved or straight galea length of beaked species, lower lip width and pollen grain volume) of eight species of *Pedicularis* in relation to wingbeat frequency, buzz frequency and buzz/wingbeat frequency ratio for *B. friseanus*

	Independent variable	Dependent variable	d.f.	Mean square	F	P
A	Wingbeat frequency	Lower lip width	1	8062.3	20.20	<0.001
		Galea straight	1	694.1	1.825	0.19
B	Buzz frequency	Wingbeat frequency	1	13868.8	16.19	<0.001
		Galea curve	1	5351.4	6.246	0.02
C	Buzz/wingbeat frequency ratio	Wingbeat frequency	1	0.176	7.862	0.009
		Galea straight	1	0.138	6.146	0.02

Bold: $P < 0.05$.

Lengoc, 1993; King and Ferguson, 1994). Kiwifruit, *Actinidia deliciosa*, is buzz-pollinated. Its pollen grains were shown to be activated by vibration in a manner that depended on the degree of hydration of the pollen grains in an anther (Corbet *et al.*, 1988; King and Lengoc, 1993; King and Ferguson, 1994). King and Ferguson (1994) showed that within an *Actinidia* anther the deepest pollen grains were mostly embedded in tapetal fluid and vibration would not shift them. In the middle regions of an anther were clumps of mostly dehydrated pollen joined by tapetal fluid droplets, and vibration of this moderately hydrated pollen could cause explosive release of individual grains or small clumps, which fragmented on contact with a surface (King and Buchmann, 1996). King and Lengoc (1993) proposed that this explosive release was due to vibration causing sudden breaking of the bridges of tapetal fluid between the grains. In the dry surface pollen in the top of the locule the fluid droplets were almost entirely dehydrated, with only very small remnants remaining. Even a slight force caused such dry

pollen to be released from the anther, and vibration at appropriate velocities resulted in a dancing movement described as 'boiling' (Corbet *et al.*, 1982; King and Lengoc, 1993).

A triboelectric hypothesis

We postulate that the boiling of dry pollen and the explosive release of partially hydrated pollen may result from the mutual repulsion of grains sharing an electrostatic charge. Particles gain electrostatic charge as a result of vibration-induced collisions with a surface (here, the anther walls) (Matsusaka *et al.*, 2010). Such charging depends on collision frequency, which is proportional to velocity (Buchmann and Hurley, 1978; Matsusaka *et al.*, 2010). When pollen grains in an anther gain enough charge to boil, they would escape from the anther if there was free passage to the outside. But if they are contained within an anther from which the only exit is a narrow terminal pore, movement resulting directly from vibration would be enhanced by boiling movement resulting from mutual repulsion of pollen grains within the anther. Pollen grains or clumps that boil within an anther would experience numerous collisions with the anther walls and with other grains or clumps, increasing their charge and perhaps fragmenting the clumps, before escaping through the pore.

When particles of powder pass along a pipe of a different material, collisions with the pipe walls cause impact-charging of the particles (gas–solids pipe flow) (p. 5802 in Matsusaka *et al.*, 2010). The longer the pipe, the higher the charge on the particles (Masuda *et al.*, 1998). The efficiency of this triboelectric charging can be increased by using spiral pipes instead of straight ones (p. 5791 in Matsusaka *et al.*, 2010), presumably by increasing collision frequency.

Charging within a vibrating anther might be maximized by plant adaptations that increase the number of collisions before grains escape from the anther. Therefore, there may be an adaptive advantage in extending a tubular poricidal anther, either by lengthening the anther or by adding a tubular extension, as in *Demosthenesia cordifolia* or *Solanum eleagnifolium* (p. 81 in Buchmann, 1983), and curving the anther or even coiling

TABLE 6. Sonication behaviour of those workers of *B. friseanus* and *B. festivus* (means of *n* individual bee means, with s.e.m.) for which pulse data were recorded in 2012, on six *Pedicularis* species

Bee species		<i>Pedicularis</i> species					
		<i>cephalantha</i>	<i>densispica</i>	<i>longiflora</i>	<i>oxycarpa</i>	<i>siphonantha</i>	<i>tricolor</i>
<i>B. friseanus</i>	Buzz frequency, Hz	309.3 (6.640) <i>n</i> = 5	286.0 (7.687) <i>n</i> = 4	318.7 (17.815) <i>n</i> = 3	333.3 (7.708) <i>n</i> = 3	290.7 (2.987) <i>n</i> = 3	263.8 (19.926) <i>n</i> = 7
	Wingbeat frequency, Hz	207.1 (13.652) <i>n</i> = 5	182.3 (8.695) <i>n</i> = 4	234.6 (8.471) <i>n</i> = 3	233.2 (3.842) <i>n</i> = 3	215.5 (11.283) <i>n</i> = 3	183.1 (4.771) <i>n</i> = 7
	Buzz/wingbeat ratio	1.525 (0.119) <i>n</i> = 5	1.575 (0.046) <i>n</i> = 4	1.357 (0.031) <i>n</i> = 3	1.431 (0.050) <i>n</i> = 3	1.356 (0.069) <i>n</i> = 3	1.435 (0.087) <i>n</i> = 7
	Seconds/pulse	0.848 (0.183) <i>n</i> = 5	0.233 (0.012) <i>n</i> = 4	0.677 (0.195) <i>n</i> = 3	0.660 (0.091) <i>n</i> = 3	0.96 (0.036) <i>n</i> = 3	0.738 (0.144) <i>n</i> = 7
	Pulses/flower	1.748 (0.186) <i>n</i> = 5	2.585 (0.474) <i>n</i> = 4	1.833 (0.095) <i>n</i> = 3	2.237 (0.391) <i>n</i> = 3	1.333 (0.049) <i>n</i> = 3	2.130 (0.509) <i>n</i> = 7
	Seconds/flower	1.350 (0.183) <i>n</i> = 5	0.600 (0.105) <i>n</i> = 4	1.217 (0.298) <i>n</i> = 3	1.51 (0.409) <i>n</i> = 3	1.283 (0.094) <i>n</i> = 3	1.231 (0.127) <i>n</i> = 7
<i>B. festivus</i>	Buzz frequency, Hz	297.2 (13.034) <i>n</i> = 8		291.1 (1.809) <i>n</i> = 4	281.6 <i>n</i> = 1		
	Wingbeat frequency, Hz	157.94 (2.937) <i>n</i> = 8		145.45 (5.271) <i>n</i> = 4	171.7 <i>n</i> = 1		
	Buzz/wingbeat ratio	1.883 (0.082) <i>n</i> = 8		2.011 (0.090) <i>n</i> = 4	1.640 <i>n</i> = 1		
	Seconds/pulse	0.516 (0.090) <i>n</i> = 8		0.453 (0.047) <i>n</i> = 4	0.420 <i>n</i> = 1		
	Pulses/flower	3.844 (0.937) <i>n</i> = 8		2.635 (0.563) <i>n</i> = 4	4.670 <i>n</i> = 1		
	Seconds/flower	1.534 (0.233) <i>n</i> = 8		1.313 (0.267) <i>n</i> = 4	1.950 <i>n</i> = 1		

TABLE 7. Analyses of variance and covariance for the relationship between the temporal patterning of pulses, wingbeat frequency and species of bees and flowers. Pulses per flower for *B. friseanus* alone were not significantly related to wingbeat frequency or flower species

	Dependent variable	Independent variable	d.f.	Mean square	<i>F</i>	<i>P</i>
A	Pulses per flower, all bees	Bee species	1	17.287	7.1193	0.01
B	Seconds per flower, all bees	Bee species	1	0.8939	3.8288	0.06
C	Seconds per pulse, all bees	Bee species	1	0.2796	3.6271	0.07
D	Seconds per flower, <i>B. friseanus</i>	Flower species	5	0.2934	3.8062	0.01
E	Seconds per pulse, <i>B. friseanus</i>	Flower species	5	0.4069	2.8373	0.06

Bold: *P* < 0.05.

it in a spiral, as in *Cochliostema odoratissima* (Fig. 5.3, p. 151 in Endress, 1994) to increase the charge on the emerging pollen grains. The length of the locule and the narrowness of the terminal pore may prevent pollen clumps from escaping until collisions have broken the bridges of tapetal fluid that hold pollen grains together, so that the clumps, now highly charged, are small enough to emerge from the pore. The same considerations apply to the beak of *Pedicularis*, the functional equivalent of the elongate locule of a poricidal anther. The beak is one of the most evolutionarily labile and morphologically diverse features of the flowers of *Pedicularis* (Ree, 2005). Perhaps the length of the beak and its curvature increase the collision rate and so increase the triboelectric charging of pollen grains during their passage from the anthers to the exit at the tip of the beak, and perhaps the narrowness of the beak and the terminal pore delay the escape of boiling pollen grains or clumps, allowing them to fragment into smaller clumps and gain higher charge before release.

Flying bees carry an electrostatic charge (Vaknin *et al.*, 2000). If the electrostatic charge on pollen causes the grains to be

attracted to a charged bee, there may be a strong adaptive advantage for the plant in increasing the charge on the grains when they are released, because that will mean that a higher proportion of the grains reach a bee, and a lower proportion are wasted. Buzzing is expected to increase the charge on a bee's body (Vaknin *et al.*, 2000), as well as that on the pollen, and might therefore improve the bee's foraging efficiency by increasing the proportion of pollen grains contacting the bee's body, even in flowers that are not adapted to buzz pollination. Buzzing necessarily results in heat production (Heinrich, 1993), so the thoracic temperature of a bee is expected to rise during buzzing. Sonication of a flower is often broken into a succession of brief pulses (e.g. de Luca and Vallejo-Marin, 2013), perhaps because a sustained period of continuous buzzing would bring the thoracic temperature above the acceptable range. The temporal pattern of buzzing on a flower may be a compromise between maximizing pollen release and avoiding dangerously high body temperatures. Alternatively, the declining yield of successive pulses may provide information telling the bee at what stage further buzzing will release little pollen.

If vibration-induced pollen activation (boiling or explosive release) results wholly or partly from triboelectric charging, the critical threshold for pollen activation is expected to be related to the velocity component of vibration.

Does the critical threshold for pollen activation depend on the velocity component of vibration?

Several studies on buzz pollination can be interpreted as showing that activation (boiling or explosive release) of pollen grains in an anther depends on the velocity element of the vibration imposed by a bee.

King and Buchmann (1996) found that vibration made pollen of *Solanum laciniatum* move vigorously, whether it was in the anthers or free, piled on a shaker table. Acceleration, A , is given by

$$A = 4\pi^2 f_v^2 D \quad (1)$$

where f_v is vibration frequency and D is displacement (p. 455 in King and Buchmann, 1996); and velocity, V , is proportional to the product of displacement and frequency (p. 105 in Buchmann, 1983; King and Buchmann, 1995; p. 452 in King and Buchmann, 1996):

$$V \propto D f_v \quad (2)$$

Hence velocity is proportional to acceleration divided by frequency (King and Buchmann, 1995):

$$V \propto A / f_v \quad (3)$$

King and Buchmann (1996) found a linear relationship between the acceleration and frequency required to initiate pollen movement, whether the pollen grains were within anthers of *S. laciniatum* or removed from the anthers and piled on a shaker table. The implication of the straight-line relationship is that movement was initiated at a constant value of acceleration/frequency, i.e. at a critical threshold velocity.

de Luca *et al.* (2013) used freshly opened flowers of *Solanum rostratum* and kept them at high humidity before use, so it is likely that the grains were clumped together with tapetal fluid. They vibrated flowers, varying frequency and 'amplitude'. The term 'amplitude' is ambiguous in this context. They said that amplitude could be measured by displacement, velocity or acceleration, and in that study they expressed amplitude in velocity units. They found that pollen emission from anthers of *S. rostratum* depended on velocity (= 'amplitude'), and that when velocity was kept constant, variation in frequency had no important effect on pollen emission.

de Luca and Vallejo-Marin (2013) used the term 'peak amplitude' to refer to 'the energy contained within a buzzing vibration', and expressed peak amplitude in acceleration units. They cited de Luca *et al.* (2013) as having shown that the amount of pollen released by vibration from anthers of *S. rostratum* increased with greater amplitude (although in that paper amplitude had been expressed in velocity units); and that 'variation in frequency had a negligible effect on the amount of pollen removed'.

Working with *Dodecatheon conjugens*, Harder and Barclay (1994) assessed the effect of vibration frequency on pollen removal for a fixed 'input energy', which they defined as the product of 'amplitude' (by which they meant displacement) and frequency. They found that pollen removal did not differ significantly among frequencies over the relevant range (up to 400 Hz). Because the product of displacement and frequency is proportional to velocity (eqn 2) (p. 105 in Buchmann, 1983), they presumably kept velocity constant while varying displacement and frequency. To keep velocity constant, any increase in frequency would have been compensated by a decrease in displacement. Again, their findings are consistent with the notion that pollen release depends on velocity.

The findings of de Luca *et al.* (2013) and Harder and Barclay (1994) have led some to conclude that variation in frequency has no important effect on pollen emission. But if pollen release depends on velocity, and velocity is proportional to the product of frequency and displacement (eqn 2), then if displacement is kept constant pollen release will depend on frequency. It follows that a bee capable of limited displacement, perhaps constrained by thoracic size and structure, must adjust its vibration frequency in order to achieve the critical velocity for pollen release.

The displacement is potentially greater in larger bees; small bees are expected to elevate buzz frequency to achieve velocity comparable to that achieved by a larger bee. Our findings support that interpretation, in that the buzz frequency was inversely related to the size (positively related to the wingbeat frequency, f_w) of the bee (Fig. 4A):

$$D \propto 1/f_w \quad (4)$$

Hence from eqn 2

$$V \propto f_v / f_w \quad (5)$$

where f_v/f_w is the buzz/wingbeat frequency ratio. The remarkably high correlation between this ratio and floral traits (Fig. 4E, F) supports the view that this ratio reflects the velocity component of the buzzing vibration.

Sonicated bees grasp the base of the beak with the mandibles (Fig. 1) as they vibrate the thorax, and pollen is emitted from the tip of the beak onto the closest part of the bee's body (Huang and Shi, 2013). If the beak tip extended beyond the bee's body, less of the pollen would be deposited on the bee. When the beak tip is positioned against the bee's body, large bees can grasp the beak close to the anthers, whereas smaller bees must grasp the beak lower down, further from the anthers, presumably reducing the efficiency of transmission of their vibration to the anthers.

Conclusions

Ours is not the only hypothesis to account for the dynamics of pollen release by sonication (Buchmann and Hurley, 1978), and the mechanism may prove to involve elements of each. But the prediction of our triboelectric hypothesis, that the critical threshold for pollen activation depends on the velocity component of vibration, is supported by the experiments of King and Buchmann (1996), de Luca *et al.* (2013) and Harder and Barclay (1994) (see above), and fits with the close correlation between the buzz/wingbeat frequency ratio and two floral traits among *Pedicularis* species. The hypothesis is not falsified, but further work, using more sophisticated methods and equipment, would be required to allow it to be accepted or rejected with confidence.

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