

1 Floral vibrations by buzz-pollinating bees
2 achieve higher frequency, velocity and
3 acceleration than flight and defence
4 vibrations

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6 David J. Pritchard ^{1,*}

7 Mario Vallejo-Marín ¹

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9 ¹ Department of Biological and Environmental Sciences, Faculty of Natural Sciences, University of

10 Stirling. FK9 4LA. Stirling, Scotland, United Kingdom

11 * Author for correspondence.

12 Correspondence: david.pritchard@stir.ac.uk

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15

16 Abstract

17 Vibrations play an important role in insect behaviour. In bees, vibrations are used in a variety of
18 contexts including communication, as a warning signal to deter predators and during pollen foraging.
19 However, little is known about how the biomechanical properties of bee vibrations vary across
20 multiple behaviours within a species. In this study, we compared the properties of vibrations
21 produced by *Bombus terrestris audax* (Hymenoptera: Apidae) workers in three contexts: during
22 flight, during defensive buzzing, and in floral vibrations produced during pollen foraging on two buzz-
23 pollinated plants (*Solanum*, Solanaceae). Using laser vibrometry, we were able to obtain contactless
24 measures of both the frequency and amplitude of the thoracic vibrations of bees across the three
25 behaviours. Despite all three types of vibrations being produced by the same power flight muscles,
26 we found clear differences in the mechanical properties of the vibrations produced in different
27 contexts. Both floral and defensive buzzes had higher frequency and amplitude velocity,
28 acceleration, and displacement than the vibrations produced during flight. Floral vibrations had the
29 highest frequency, amplitude velocity and acceleration of all the behaviours studied. Vibration
30 amplitude, and in particular acceleration, of floral vibrations has been suggested as the key property
31 for removing pollen from buzz-pollinated anthers. By increasing frequency and amplitude velocity
32 and acceleration of their vibrations during vibratory pollen collection, foraging bees may be able to
33 maximise pollen removal from flowers, although their foraging decisions are likely to be influenced
34 by the presumably high cost of producing floral vibrations.

35

36 **Keywords:** Apidae, bee behaviour, biomechanics, *Bombus*, buzz pollination, energetic costs, flight,
37 poricidal anthers, *Solanum*.

38 Introduction

39 Vibrations play an essential role in the natural behaviour of animals, particularly, among
40 invertebrates. For example, spiders and antlions use vibrations produced by prey during hunting
41 (Guillette et al., 2009; Mencinger-Vračko & Devetak, 2008; Nakata, 2010), and larval leafminers use
42 vibrations to detect and avoid parasitoid wasps (Djemai et al., 2001). Animal vibrations can be
43 transmitted both through the air (sound) and through the underlying substrate (most often plant
44 tissue) as substrate-borne vibrations (Cocroft & Rodríguez, 2005). The substrate-borne component
45 of vibrations can be particularly important in some contexts such as during insect communication
46 because vibrations produced by small animals can be more efficiently transmitted through the
47 substrate than through air (i.e. as sound) (Barth et al., 2005; Cocroft and Rodríguez, 2005; Mortimer,
48 2017).

49 Most studies of insect vibrations have focussed on vibrations produced for communication
50 or as a by-product of flight (Hill et al., 2019; Tercel et al., 2018). But insects can use vibrations for
51 much more than communication and locomotion. Among bees, vibrations play a particularly
52 multifaceted role. For example, bees not only use vibrations to communicate with their nest mates
53 (Barth et al., 2005) and as a warning or defence mechanism against potential predators (Hrnčir et al.
54 2008; Barth et al., 2005), but also during nest construction (Rosenheim, 1987), and as a foraging tool
55 to harvest pollen from certain flowers (Macior, 1962; Thorp, 2000; Vallejo-Marín, 2019). For
56 example, substrate-borne vibrations are one of the ways in which some bees can rapidly dislodge
57 and collect pollen on flowers with poricidal anthers (anthers that release pollen through small pores
58 or slits; Buchmann, 1983). The ability to use vibrations during pollen harvesting occurs in
59 approximately 58% of all bee (Anthophila) species including 15% of genera in all bee families
60 (Cardinal et al., 2018), and buzz-pollination (pollination using vibrations) is associated with more
61 than 20,000 species of flowering plants (Buchmann, 1983; De Luca & Vallejo-Marín, 2013). Despite
62 the widespread use of vibrations across diverse behavioural contexts, including during buzz

63 pollination, we still know relatively little about the extent to which vibrational properties vary within
64 the same species and across behaviours.

65 In bees, the same mechanism that drives the wings during flight is responsible of producing
66 vibrations used during communication, defence and buzz pollination. Vibrations are produced by
67 cyclical deformations of the bee's thorax caused by the alternate contraction of dorsal longitudinal
68 and dorso-ventral power flight muscles (Hedenström, 2014). These contractions are not
69 synchronised with nerve impulses, instead bee flight muscles are "stretch-activated", with the
70 stretching of one of the antagonistic pairs of muscles stimulating the contraction of the other. This
71 cycle of stretching and contraction creates a relatively self-sustaining series of cyclical thorax
72 contractions along longitudinal and ventral axes (Dickinson, 2006; Josephson et al., 2000), with nerve
73 impulses mostly working to maintain this cycle or make broad-scale changes such as an increase in
74 power (Gordon & Dickinson, 2006).

75 Despite sharing a common production mechanism (thoracic power flight muscles), flight and
76 non-flight vibrations in bees have clearly different vibrational properties. Non-flight vibrations are
77 produced with the wings folded, effectively uncoupling power flight muscle contraction and
78 wingbeat (King et al., 1996). For a given bee species, non-flight vibrations have higher frequencies
79 than those produced during flight (Barth et al., 2005; De Luca et al., 2019; Hrncir et al., 2008; King &
80 Buchmann, 2003), in part due to reduced drag from the wings as well as increased tension in the
81 thoracic muscles (Hrncir et al., 2008; King et al., 1996). In contrast, non-flight vibrations produced in
82 different contexts are superficially very similar. Both defence and floral vibrations are produced with
83 folded wings and it is not clear to what extent non-flight thoracic vibrations have different properties
84 to one another. Few studies have compared non-flight vibrations produced in different contexts on
85 the same bee species. Hrncir et al. (2008) found that the frequency of vibrations produced by the
86 tropical stingless bee, *Melipona quadrifasciata* Le Peletier (1836) (Apidae), during defence buzzes is
87 approximately 60% of the frequency of vibrations used to communicate between foragers (350 vs.

88 487 Hz, respectively). In bumblebees (*Bombus spp.* Latreille 1802), comparison of two European
89 species found frequency differences in non-flight vibrations, namely defence and floral buzzes.
90 However, the direction and size of the difference in frequency between defence and floral buzzes
91 differed between the two bumblebee species (De Luca et al., 2014). While non-flight vibrations in
92 bees are a potentially useful system for understanding the evolution and diversification of vibratory
93 behaviours, clearly, more work is needed to characterise the exact differences between non-flight
94 vibrations in different contexts.

95 Comparing the properties of vibrations produced on different behavioural contexts is
96 technically challenging. Traditionally, substrate-borne vibrations produced by bees have been
97 studied indirectly by recording the airborne component of the vibration using acoustic recorders.
98 Yet, recent work indicates that although frequency components are reliably inferred from either
99 acoustic or substrate-borne measurements, the magnitude of substrate-borne vibrations are poorly
100 correlated with the magnitude of their acoustic component (De Luca et al., 2018). This may be
101 because small invertebrates are poor acoustic transducers (De Luca et al., 2018), a view that is
102 consistent with the fact that most insect communication occurs through a plant substrate, rather
103 than through airborne sound (Cocroft & Rodríguez, 2005). This is one reason why most of the
104 previous work comparing the vibration properties of different bee behaviours has been focused on
105 acoustically measured frequency differences, with relatively few studies attempting to measure both
106 frequency and amplitude (acceleration, velocity or displacement) components (Nieh and Tautz,
107 2000; Hrnčir et al., 2008). To get a more complete view of how vibrations differ across bee
108 behaviours, it is necessary to capture both frequencies and amplitudes components (Vallejo-Marín,
109 2019). Vibration amplitude can be experimentally measured using vibration transducers such as
110 accelerometers or laser vibrometers (Cocroft & Rodríguez, 2005). A full characterisation of
111 substrate-borne vibrations is particularly important in the context of buzz pollination because
112 biophysical models of poricidal anthers (Buchmann & Hurley, 1978), as well experimental tests with

113 artificial buzzes, suggest that vibration amplitude, rather than frequency, is a key determinant of the
114 rate of pollen ejection from flowers (De Luca et al., 2013; Rosi-Denadai et al., 2018).

115 In this study, we characterised for the first time, the extent to which a single species of
116 bumblebee can modify the properties of their vibrations across multiple behaviours. Rather than just
117 comparing flight and non-flight vibrations, we used accelerometers and laser vibrometry to directly
118 measure the vibrational properties of buzzes produced by bumblebees (*Bombus terrestris* ssp.
119 *audax*, (Harris 1776); hereafter *B. audax*) both during flight and in two different non-flight
120 behavioural contexts: defence and floral vibrations. In addition, we compare the floral vibrations
121 produced by bees on two different buzz-pollinated plant species (*Solanum rostratum* Dunal and *S.*
122 *citrullifolium* (A. Braun) Nieuwl., Section *Androceras*, Solanaceae). Previous work has shown
123 conflicting results on the extent to which bumblebees change the vibrations produced during floral
124 visitation (floral vibrations), with some studies showing differences between flowers (Switzer and
125 Combes, 2017) or with experience (Morgan et al., 2016; Switzer et al., 2019) and others showing
126 more limited flexibility (Russell et al., 2016b). However, while other studies of bee vibrations have
127 used non-contact methods (laser vibrometry) to look at differences in vibration properties (Conrad
128 and Ayasse, 2015; Conrad and Ayasse, 2019), few studies to date have used these methods to
129 examine floral vibrations directly on bees (Nunes-Silva et al., 2013). Our study addresses three
130 specific questions: 1) What are the main differences in the vibrations produced by bumblebees
131 across different behaviours? 2) To what extent floral vibrations produced by the bee depends on the
132 species of flower being visited? 3) Do the characteristics of vibrations depend on bees'
133 morphological traits such as size?

134 Materials and methods

135 Study system

136 Bees

137 We used two colonies of the buff-tailed bumblebee, *Bombus terrestris audax* (Koppert, Agralan Ltd,
138 Wiltshire, UK). Each colony had access to *ad libitum* “nectar” solution (Koppert) within the colony.
139 Each colony was attached to a flight arena (122 × 100 × 37 cm), illuminated with an LED light panel
140 (59.5 × 59.5 cm, 48 W Daylight; Opus Lighting Technology, Birmingham, UK) and maintained on a
141 12h:12h supplemental light:dark cycle. The ambient temperature was 20-23°C and humidity was 50-
142 60% RH. In each arena, bees were also provided with a 1M sucrose solution, *ad libitum*, from three
143 feeders in each colony, as well as eight inflorescences (four *Solanum rostratum*, four *S. citrullifolium*)
144 every two days.

145 Plants

146 We tested floral vibrations on two closely related species from the genus *Solanum* (Solanaceae).
147 *Solanum rostratum* and *Solanum citrullifolium* are both nectarless species, which attract and reward
148 pollinators solely with pollen. In common with other *Solanum* species, *S. rostratum* and *S.*
149 *citrullifolium* have poricidal anthers, which requires pollinators to vibrate the anthers to release
150 pollen. Unlike some other *Solanum* species, *S. rostratum* and *S. citrullifolium* are both
151 heterantherous, with bees primarily focussing their attention on “feeding anthers” presented at the
152 centre of the flower, while a single, rarely visited “pollination anther” deposits pollen on the visiting
153 bee. *Solanum* species are a classic system for the study of buzz pollination (e.g. Buchmann & Cane,
154 1989; King & Buchmann, 1996), and *S. rostratum* and *S. citrullifolium* have been directly compared in
155 a previous study which identified apparent difference in the coupling factors of these species
156 (Arroyo-Correa et al., 2019). Vibrations applied to *S. rostratum* show less attenuation than vibrations
157 applied to *S. citrullifolium*, making this pair an ideal comparison for the effect of bee-produced
158 vibrations on flowers.

159 *S. rostratum* and *S. citrullifolium* plants were grown from seed at the University of Stirling
160 research glasshouses, using the method described in Vallejo-Marín et al. (2014). Seeds of *S.*
161 *rostratum* were collected in Mexico (20.901°N, 100.705°W; accessions 10s77, 10s81, 10s82) and
162 seeds of *S. citrullifolium* were obtained from self-fertilised fruits (accession 199) grown from seeds
163 obtained from Radboud University's seed collection (accession 894750197). For daily flower
164 provision for bees, inflorescences were placed in water-soaked Ideal Floral Foam (Oasis Floral
165 Products, Washington, UK) in plastic containers. For experiments, we used a single flower, cut 2-3cm
166 below the calyx.

167 Recording of floral vibrations

168 To facilitate the recording of bee vibrations using laser vibrometry, we tagged individual bees with a
169 small (2mm²) piece of reflective tape placed in the dorsal part of the thorax. Bees buzzing on flowers
170 in the flight cages were captured, placed in a freezer at -26°C for seven minutes, and tagged with
171 reflective tape using Loctite UltraControl instant adhesive (Henkel Limited, Winsford, UK). After
172 being at room temperature, bees resumed normal activity after approximately 7-10 minutes and
173 were released back into the colony.

174 At least 24 hours after being tagged, bees were allowed to visit flowers in the arena and a
175 tagged bee which was actively buzzing flower was collected from flowers in the flight cage and
176 released onto a single flower of either *S. rostratum* or *S. citrullifolium* in the test arena. The flower
177 species were chosen so that each colony received the same number of flowers from each plant
178 species. The vibrations produced by the bee were recorded simultaneously in two ways. First, we
179 measured vibrations produced in the bee's thorax using a laser vibrometer (PDV 100, Polytec,
180 Coventry, UK). Laser vibrometry provides a direct, contactless measure of the vibrations produced by
181 the bee. Vibrations measured with the laser were sampled at a rate of 10240 Hz using a low pass
182 filter of 5Hz, and a maximum velocity range of either 100 mm/s (for bees 1-14) or 500 mm/s (for
183 bees 15-32). The laser vibrometer was placed approximately 20cm away from the flower and aimed

184 at the reflective tag on the bee's thorax. Second, we used an accelerometer (352C23, 0.2g; PCB
185 Piezotronics) to record the vibrations transmitted from the bee to the flower (Arroyo-Correa et al.,
186 2018). The accelerometer was attached to the calyx at the base of the flower being vibrated by the
187 bee using a 5mm x 0.35mm pin made from an entomological pin (Austerlitz, Size 0) and glued to the
188 accelerometer with instant adhesive as described in Arroyo-Correa et al. (2018). The accelerometer
189 and laser were set to register along the same axis of movement.

190 Both laser vibrometer and accelerometer data were simultaneously recorded and time-
191 stamped using Data Acquisition System (cRIO model 9040 with the C series module NI 9250;
192 National Instruments, Newbury, UK) using a custom-made LabView (National Instruments) program
193 (available upon request). While the bee buzzed the flower, data were recorded during two seconds
194 at a sampling rate of 10240 Hz and saved to a file. After collecting 5-10 buzzes for each bee, the bee
195 was caught in a 30mL plastic container (201150; Greiner, Gloucestershire, UK), and euthanised by
196 being placed in -26 freezer for 48 hours. In total, we collected data for 16 bees from two colonies,
197 eight on each flower species. For each bee we recorded analysed an average of 6.13 buzzes (N = 98
198 buzzes from 16 bees).

199 Recording of defence and flight vibrations

200 For the recording of flight and defence buzzes bees were selected at random from the flight box. As
201 for the flower buzzing, bees were immobilised by being placed in the freezer for seven minutes. In
202 addition to gluing a 2mm² reflective tag to the scutum, immobile bees were also tethered to the
203 apparatus for recording defence and flight buzzes, similar to that used by Hrncir et al. (2008). The
204 neck of the bee was held by a loop of fine nylon string threaded through a needle and attached to a
205 syringe secured by a clamp (Figure 1). After 7-10 minutes, the tethered bee had returned to regular
206 activity levels and we continued with data collection.

207 To record both flight and defence buzzes, the laser vibrometer was placed above the bee
208 and aimed at the tag on the bee's thorax. The laser beam was perpendicular to the platform on

209 which the bee was tethered. Defence and flight vibrations measured with the laser were sampled at
210 a rate of 10240 Hz using a low pass filter of 5Hz, and a maximum velocity range of 500 mm/s. To
211 induce defence buzzes, the tethered bees were gently squeezed along the sides using featherweight
212 forceps. To record flight buzzes, the platform underneath the tethered bee fell away inducing the
213 bee to start flight activity (Hrncir et al., 2008). As before, vibration data was recorded through the
214 cRIO data acquisition system using a custom LabVIEW program, which collected two seconds of data
215 at a time at a sampling rate of 10240 Hz, with a low pass filter of 5Hz and a velocity range of 500
216 mm/s. Flight and defence buzzes were recorded from 20 bees in total, with defence and flight buzzes
217 captured from all bees. To avoid order effects, 10 of the bees had defence buzzes collected first and
218 10 had flight collected first. Following recording, tethered bees were immobilised again by being
219 placed in the freezer, removed from the tether, placed in a plastic container, and euthanised in the -
220 26°C freezer. For each bee, we analysed an average of 5.6 flight vibrations (n = 112 vibrations from
221 20 bees) and 6.8 defence buzzes (n = 136 from 20 bees).

222 Bee size

223 Bee size was approximated using intertegular distance (ITD), the distance between the tegulae at the
224 base of the wings (Cane, 1987). We measured ITD using a digital photograph of euthanised bees
225 taken with a dissecting microscope (MZ6, Leica Microsystems, Milton Keynes, UK) (Figure S1), and
226 analysed with the *Fiji* distribution of *ImageJ* (Schindelin et al., 2012).

227 Data Analysis

228 Analysing vibrations

229 We used a section of each recorded vibration for analysis (Figure 2). For floral buzzes, we selected a
230 section of each recording that successfully captured both laser and accelerometer sensors. The
231 sensor data (time series with voltage units) were converted from voltage to either velocity (laser) or
232 acceleration (accelerometer) using the factory-provided conversion factors for each sensor. We
233 zero-centred the data by subtracting the mean amplitude from each value and applied an 80-5000

234 Hz band-pass filter and a Hamming window (window length = 512), using the *fir* function in the *R*
235 package *seewave* (Sueur et al., 2008). The acceleration data were converted to velocity by numerical
236 integration using the *cumtrapz* function in the *pracma* package (Borchers, 2019), and the band-pass
237 filter was applied again. The fundamental frequency of the analysed vibration was obtained with the
238 *fund* function, calculated over the entire sample and setting a maximum frequency to 1000 Hz. Peak
239 amplitude velocity for each vibration segment was calculated from the amplitude envelope
240 calculated using the *env* function with a mean sliding window of length 2 and an overlap of 75%. All
241 analyses were done in *R* version 3.6.0 (R Core Team, 2019)

242 Transmission of bee vibrations through flowers

243 To quantify the extent to which the vibrations produced by bees differ from those measured in the
244 flower itself, we calculated King's coupling factor (King, 1993). The bee's coupling factor (K_{bee}) was
245 calculated by dividing the root mean squared (RMS) amplitude velocity of the vibration produced by
246 the bee by the RMS amplitude velocity recorded by the accelerometer placed in the flower's calyx
247 (Arroyo-Correa et al. 2019). We also calculated King's coupling for vibrations produced by a
248 mechanical calibrated shaker (Handheld shaker model 394C06, PCB Piezotronics). The calibrated
249 shaker produces a vibration of constant properties (frequency = 159.2Hz, RMS amplitude velocity =
250 9.8 mm s^{-1}) that are transmitted to a small metal plate at one end of the instrument. The metal plate
251 of the calibrated shaker was firmly pushed against the feeding anthers of the flower, and we
252 recorded four to five samples of two seconds each using the data acquisition system described
253 above (*Analysing Vibrations*). For each flower, we selected one clean recording, converted voltage to
254 velocity as described above, and obtained King's coupling factor for the shaker (K_{shaker}) using the
255 ratio between expected and observed RMS velocity. Measuring both K_{bee} and K_{shaker} allowed us to
256 compare the difference in the efficiency with which a bee and a mechanical shaker transmit
257 vibrations to the flower.

Properties of vibrations in bumblebees

258 Statistical analyses

259 To compare the properties of vibrations in different contexts we used linear mixed effect models
260 using either peak velocity or fundamental frequency as response variables, buzz type
261 (flight/defence/floral) and intertegular distance as explanatory variables, and bee identity as a
262 random effect. In addition to peak velocity and frequency, which were measured directly, we also
263 used these measures to derive the displacement amplitude (in mm) and acceleration (in mm/s²) of
264 the vibration. As with velocity, we analysed the peak recordings of each of these measures with
265 linear mixed effect models, with buzz type and intertegular distance as explanatory variables and
266 bee identity as a random effect. To compare the properties of floral vibrations on different *Solanum*
267 species, we employed linear mixed effect models, using either laser-recorded peak velocity, laser-
268 recorded fundamental frequency, accelerometer-recorded peak velocity or accelerometer-recorded
269 fundamental frequency as response variables, flower species and intertegular distance as
270 explanatory variables, and bee identity as a random effect. Finally, to compare the effect of flower
271 species and recording method on coupling factors, we used a linear mixed effect model with
272 coupling factor as a response variable, flower species, intertegular distance, and vibration method
273 (bee vs artificial) as explanatory variables, and bee ID as a random effect. All analyses were
274 performed using *lme4* (Bates et al., 2015) to estimate parameters and *lmerTest* (Kuznetsova et al.,
275 2017) to assess statistical significance.

276 Ethical approval

277 These experiments were approved by the Animal Welfare and Ethical Review Board of the University
278 of Stirling.

279 Data availability

280 Data and code will be deposited in Dryad with information given in the final manuscript.

281 Results

282 Comparison of buzzes produced in different behavioural contexts

283 The vibrations produced during flight, defence and pollen extraction differ significantly in properties
 284 including fundamental frequency and peak amplitude velocity (Table 1). The peak amplitude velocity
 285 of floral buzzes (262.85 ± 9.52 mm/s) was significantly higher than both defence (194.85 ± 6.12
 286 mm/s) and flight buzzes (57.29 ± 1.28 mm/s; Figure 3A, Table 1). We found no significant effect of
 287 bee size on peak amplitude velocity (Table 1). Floral buzzes also had significantly higher frequencies
 288 (313.09 ± 2.63 Hz) than both defence (236.32 ± 4.29 Hz) and flight buzzes (136.95 ± 1.73 Hz) (Figure
 289 3B). We also detected an interaction between bee size and buzz type with larger bees achieving
 290 higher frequency defence buzzes and lower frequency flower and flight buzzes than smaller bees
 291 (Table 2). The differences in peak amplitude velocity across the three behaviours observed here
 292 extended to peak amplitude acceleration, with floral buzzes achieving higher accelerations (517.77 m
 293 $s^{-2} \pm 19.40$), than defence (297.41 m $s^{-2} \pm 11.96$), and flight vibrations (49.43 m $s^{-2} \pm 1.34$) (Figure 3D).
 294 In contrast, the peak amplitude displacement of floral (0.27 mm ± 0.009) and defence buzzes (0.27
 295 mm ± 0.007) were similar, although both greater than the displacement amplitude of flight
 296 vibrations (0.14 mm ± 0.005) (Figure 3C).

297 Floral buzzes

298 Our analyses of the vibrations produced by bees while visiting flowers (floral buzzes) shows that only
 299 some of the properties of these vibrations depend on whether they are recorded on the bee or on
 300 the flower (Figure 4). The magnitude of vibrations recorded directly on the bee had considerably
 301 higher peak velocity amplitudes (273.56 ± 12.49 and 247.34 ± 14.53 mm/s for *S. rostratum* and *S.*
 302 *citrullifolium* respectively) than those vibrations measured on the flower (36.61 ± 2.30 and $19.20 \pm$
 303 1.03 mm/s for *S. rostratum* and *S. citrullifolium*, respectively; Figure 5A, Table 2). In contrast, the
 304 fundamental frequency of the floral vibrations was similar whether recorded directly from the bee
 305 (313.16 Hz ± 2.86 and 312.09 Hz ± 4.99 Hz for *S. rostratum* and *S. citrullifolium*, respectively) or

306 indirectly via the accelerometer on the flower ($312.70 \text{ Hz} \pm 2.92$ and $313.16 \text{ Hz} \pm 4.81$ for *S.*
307 *rostratum* and *S. citrullifolium*, respectively; Figure 5B, Table 2). Interestingly, we observed that
308 vibrations measured on the bee contained more harmonics (*S. citrullifolium*: 10.75 ± 0.38 ; *S.*
309 *rostratum*: 11.34 ± 0.35) than those observed on vibrations measured on the flower (*S. citrullifolium*:
310 3.65 ± 0.27 ; *S. rostratum*: 2.57 ± 0.20) (Figure 4).

311 Plant species did not significantly affect the frequency or peak amplitude velocity of floral
312 vibrations (but see section *Transmission of vibrations through flowers* for differences in the
313 transmission of vibrations from bee to flower in the two *Solanum* species). Bee size (intertegular
314 distance) was negatively associated with fundamental frequency of floral vibrations (Figure 5C),
315 while bee size had no effect on their peak amplitude velocity (Table 2). We found no statistically
316 significant interaction between bee size and plant species on either frequency or peak amplitude
317 velocity of floral vibrations.

318 Transmission of vibrations through flowers

319 To analyse the effect of plant species on the transmission of floral vibrations through the flower, we
320 compared King's coupling factor (K , the ratio of vibration magnitude produced to vibration received)
321 for the two *Solanum* species. We found that *S. rostratum* had a significantly lower coupling factor
322 ($K_{bee} = 5.64 \pm 0.61$, $K_{shaker} = 5.95 \pm 1.77$; mean \pm SE) than *S. citrullifolium* ($K_{bee} = 9.92 \pm 0.97$, $K_{shaker} =$
323 8.93 ± 1.97 ; Table 3, Figure 6). Our analysis showed no difference within plant species between
324 coupling factors calculated from either bee floral buzzes (K_{bee}) or synthetic vibrations applied with
325 the calibrated shaker (K_{shaker}) (Table 3), although K_{bee} is less variable than K_{shaker} (Figure 6). We did not
326 find an effect of bee size on coupling factor (Table 3).

327 Discussion

328 Bumblebees and other buzz-pollinating bees present a unique opportunity for research on insect
329 vibrations. In addition to producing vibrations during locomotion and as a signal to predators or

330 conspecifics, the two forms of thoracic vibrations most commonly studied in bees and other insects,
331 buzz-pollinating bees also use vibrations to forage. While the posture of bees during floral buzzes
332 and defence buzzes are very similar, with both requiring the wings folded back over the body, the
333 functions of these two buzzes are very different, making them a useful comparison for
334 understanding how function might influence the properties of bee vibrations. In this study we
335 directly compared these different types of vibrations within a single species of bumblebee, not only
336 comparing flight and non-flight vibrations, but also characterising different types of non-flight
337 vibrations. Our results show clear differences in biomechanical properties of defence and floral
338 buzzing, as well as differences between these vibrations and those produced during flight. In
339 addition to differences between different behaviours, we also found that the species of plant being
340 vibrated and the size of the bee, affected the properties of the floral vibrations experienced by
341 plants.

342 Floral vibrations and bee size

343 Our results are consistent with previous work showing that plant species differ in their transmission
344 of floral vibrations (King 1993; Arroyo-Correa et al., 2019). Between the two studied plant species,
345 we found that *Solanum rostratum* is better at transmitting vibrations applied on the anthers to other
346 parts of the flower than *S. citrullifolium*, as shown by its lower coupling factor (cf. Arroyo-Correa et
347 al., 2019). Interestingly, the coupling factor calculated using synthetic vibrations applied with a metal
348 plate and the one calculated using vibrations applied by live bees were similar, suggesting that fine
349 floral manipulation by the bee during buzzing has little effect on the vibrations transmitted to other
350 parts of the flower. Further analyses of the biomechanical properties of flowers are required to
351 determine the mechanism responsible for the different coupling factors observed here and in
352 previous studies.

353 We found little evidence that the magnitude of floral, flight and defence buzzes can be
354 explained by the range of bee size variation observed within a single species of bumblebee. In

355 contrast, bee size was negatively associated with frequency of floral and flight buzzes but positively
356 with defence buzzes. The frequency of flight vibrations in bees is usually negatively associated with
357 size both within (this study) and across species (De Luca et al., 2019). For floral vibrations, the
358 association between frequency and size seems to vary (reviewed in De Luca et al., 2019), ranging
359 from negative, as in our study on *B. terrestris audax*, to positive (Arroyo-Correa et al. 2019) to no
360 detectable relationship both within species (De Luca et al., 2013; De Luca et al. 2014, Nunes et al.
361 2013) and across multiple species (De Luca et al., 2019; Rosi-Denadai et al., 2018). Moreover, the
362 relationship between the frequency of floral buzzes and bee size within species may further depend
363 on the metric of bee size used (Corbet & Huang, 2014; Switzer & Combes, 2017). Taken together this
364 body of work suggests that differences in size are not sufficient to explain variation in floral buzzes
365 during buzz pollination.

366 Differences among buzz types

367 We found that bumblebees vibrating flowers produce higher accelerations than in other behaviours,
368 and much higher than previously thought. The floral vibrations measured in this experiment were on
369 average 500 m/s^2 , more than 2-3 times what Arroyo Correa et al. (2019) and King (1993) calculated
370 after measuring floral buzzing from the plant and correcting with the corresponding coupling factor.
371 Despite this, our measurements for frequency and velocity, from which acceleration was calculated,
372 were consistent with those found by other studies looking at flying, defence buzzing, and flower
373 buzzing bees (Nunes-Silva et al., 2003, King 1993). Floral buzzes appear to be characterised by higher
374 accelerations, velocities, and frequencies, than defence buzzes. And both floral and defence buzzes
375 have higher accelerations, velocities, displacement amplitude and frequencies, than are produced
376 during flight. The key question raised by our results, then, is why are the properties of floral, defence
377 and flight vibrations so different to one another? This question can be addressed in two ways: 1) by
378 considering how the mechanisms underlying these vibrations might differ across behaviours; and 2)
379 how the function of the behaviour might select for particular vibration properties.

380 Mechanisms of bee vibrations

381 All of the vibrations we measured in this study were produced by contractions of the dorsal
382 longitudinal and dorso-ventral flight muscles in the thorax. The fact that these vibrations all share a
383 common mechanisms could mean that something other than the muscles might be responsible for
384 the differences we observed. One early suggestion was whether the decoupling of the wings from
385 the flight muscles during non-flight vibrations (defence, floral buzzes) changed the resonant
386 properties of the thorax and led to higher frequencies. It is plausible that the deployment of the
387 wings could lower the frequency of the vibrations, wings produce drag and inertia, which is one
388 reason why insects with larger wing have a lower wingbeat frequency (e.g. Greenewalt, 1962; Joos
389 et al., 1991). When insect wings are cut shorter the frequency of flight increases (Hrncir et al., 2008;
390 Roeder, 1951). While wing deployment can explain the different between flight and non-flight
391 vibrations, it cannot explain the differences between the two non-flight vibrations (floral and
392 defence buzzes), where the wings remained folded and the mass of the system remains unchanged.

393 Instead of the mechanical effect of the wings, differences between non-flight vibrations
394 could be the result of differences in muscle activity, either in terms of increasing muscle power or by
395 changing the stiffness and resonant properties of the thorax. Although bumblebee flight muscles are
396 stretch activated, and so do not contract in time with motor neuron firing, studies of similar muscles
397 in *Drosophila* show that increasing the frequency of firing increases the Ca^{2+} concentration in the
398 flight muscles, resulting in more powerful contractions (Dickinson et al., 1998; Gordon & Dickinson,
399 2006; Lehmann & Bartussek, 2017; Wang et al., 2011). Bees could also use other muscles to stiffen
400 the thorax, changing its resonant properties, altering the frequency at which the cycle of stretch-
401 activated contractions reaches equilibrium (Nachtigall & Wilson, 1967). Although these mechanisms
402 have yet to be studied in bees, neurophysiological studies of bee flight muscles have found
403 differences between flight and non-flight vibrations (Esch & Goller, 1991; King et al., 1996), which
404 might also explain differences between non-flight vibrations. During flight, both the dorso-ventral
405 and dorsal longitudinal muscles sets are stimulated equally, whereas during defensive buzzes the

406 dorsal longitudinal muscles are stimulated at twice the rate as the dorso-ventral muscles (King et al.
407 1996). If, for example, the increased difference in activation between the flight muscles sets is
408 responsible for the increased frequency of non-flight vibrations, then we might expect the difference
409 in excitation between the muscle sets to be even more extreme during floral buzzes than during
410 defence buzzes. By comparing the mechanisms underlying floral buzzes, defence buzzes, and flight,
411 in this way, we can begin to understand how bees use changes in muscular activity and associated
412 shifts in the resonant properties of the bee's body, to adjust the mechanical properties of their
413 vibrations.

414 Function of bee vibrations

415 In addition to considering differences in the actions of the muscles, another approach to thinking
416 about *why* the muscles produce vibrations with these particular properties is to consider how what
417 properties might best serve these functions. In vibratory communication, for example, the
418 properties of the signalling environment, such as the degree of frequency filtering, determine the
419 "best" vibratory properties to transmit information from producer to receiver (Cocroft & Rodríguez,
420 2005). Similar factors could influence the "best" properties for defence buzzes. Like the vibratory
421 signals studied in other insect species, the function of a defence buzz is to transmit information from
422 the producer (the bee) to a receiver (the predator). This information is effective; defence or alarm
423 sounds produced by insects, including bumblebees, have been shown to reduce or slow down
424 predator attacks (Masters, 1979; Moore & Hassall, 2016). The effectiveness of defence buzzes is
425 likely affected by the properties of the vibration itself. Although, in our experiment, we found that
426 defence buzzes were on average of lower frequency, peak amplitude velocity and peak amplitude
427 acceleration than floral buzzes, these properties do not correlate with what is likely a more
428 important property of a warning signal: volume (De Luca et al., 2018). A previous comparison of the
429 acoustic properties of defence and floral buzzes found that defence buzzes were significantly louder
430 than floral buzzes (De Luca et al., 2014), and it is possible that the lower frequency or amplitude of
431 the bee's vibrations during defence buzzing might actually increase the perceived volume of the buzz

432 by predators. A lower frequency and velocity vibration may also be beneficial for the bee as it might
433 be less energetically costly than the higher frequency and velocity floral buzz. Although the costs of
434 buzzing by bees have only been measured for a handful of behaviours (Kammer & Heinrich, 1974;
435 Heinrich, 1975), increasing the frequency and amplitude of vibrations **could** carry a significant cost.
436 **For instance, in the carpenter bee, *Xylocopa varipuncta* Patton, increases in the frequency and**
437 **amplitude of their wingbeats when flying in less dense gases, are associated with increases in their**
438 **metabolic rate by over a third** (Roberts et al., 2004). By using lower frequency and velocity
439 vibrations, bumblebees might be able to perform defence buzzes for longer, increasing their
440 effectiveness against predators.

441 Unlike defence buzzes, the primary function of floral buzzes is not to transmit information to
442 receivers but to shake pollen loose from flowers. Pollen is essential for larval nutrition (Westerkamp,
443 1996), and bumblebees possess many specialisations to assist in pollen collection, from
444 morphological features such as corbiculae (Thorp, 1979), to behaviour specialisations, including
445 optimising pollen collection (Rasheed & Harder, 1997), rejecting flowers that appear empty of pollen
446 (Buchmann & Cane, 1989; Harder, 1990), and modifying their buzzes in response to the presence or
447 absence of pollen (Russell et al., 2016; Switzer et al., 2019). It is possible that the properties of floral
448 buzzes are also tuned to maximise the pollen collected from poricidal anthers. If that was the case,
449 we would expect the properties that defined floral buzzes in this study, high frequency, velocity, and
450 acceleration, to correlate with the vibration properties which release the most pollen. Studies with
451 artificial shakers have subjected buzz-pollinated flowers to a broad array of vibrations to determine
452 what kinds of vibration release the most pollen (De Luca et al., 2013; Harder & Barclay, 1994; Rosi-
453 Denadai et al., 2018). Although the frequency of floral buzzes appears very consistent across studies,
454 frequency does not appear to determine how much pollen is released from anthers. Instead, as we
455 observed, higher frequencies may result in higher velocities and accelerations, and it is these
456 properties which most determine how much pollen an anther releases (De Luca et al., 2013; Rosi-
457 Denadai et al., 2018). The effect of increasing the velocity or acceleration of floral buzzes on pollen

458 release can be dramatic. De Luca et al. (2013) for example, found that for a floral buzz lasting for one
459 second, doubling the velocity of the buzz led to four times as much pollen being released. Rosi-
460 Denadai et al. (2018) found a similar effect for acceleration – vibrations with a similar acceleration to
461 the floral buzzes we recorded (500 m/s^2) released more than three times as much pollen as
462 vibrations matching the flight vibrations we recorded (100 m/s^2), and twice as much as vibrations
463 matching the defence buzzes (300 m/s^2). The accelerations we recorded from floral buzzes,
464 therefore, are what might be expected from vibrations tuned to maximise pollen release. Producing
465 high acceleration floral buzzes, however, is likely to have come with a cost. Although it is not clear
466 exactly how costly these floral buzzes might be, as no-one has yet measured the metabolic cost of
467 floral buzzing, it has been suggested that bees work to maximise the efficiency of their pollen
468 collection (Rasheed & Harder, 1997). Their foraging decisions are therefore not just based on
469 maximising the pollen they collect, but also based on the potential cost. If floral buzzing exerts a
470 significant cost on bees, this cost might play an important role in their decisions about where and
471 when to forage on buzz-pollinated flowers (Stephens, 2008).

472 Conclusion

473 Our results, demonstrate clear differences between the vibrations produced by bumblebees in
474 different contexts. In addition to the expected differences between flight and non-flight vibrations
475 (De Luca et al. 2019), which can be partly attributed to wing deployment and different postures
476 resulting in physical differences in drag and resonance, we also found equally sizable differences
477 between floral and defence vibrations, in which the wings remained undeployed and posture is
478 similar. These differences between non-flight vibrations open up larger questions about the
479 mechanisms and evolution of insect vibrations. Currently the mechanisms which control the
480 properties of thoracic vibrations have only been studied in a handful of contexts (Esch & Goller,
481 1991; King et al., 1996), with most of what we know coming from studies of flight control in
482 *Drosophila* (Lehmann & Bartussek, 2017; Lindsay et al., 2017). The vibrations that individual

483 bumblebees produce in different contexts exhibit stark but reliable differences in their properties,
484 providing a model to better understand how individual insects control the properties of the
485 vibrations they produce. By identifying homologous mechanisms as well as outlining possible
486 constraints on how insect vibrations respond to selection, investigating the mechanisms of
487 bumblebee vibrations can also tell us more about how these behaviours evolve. But to understand
488 how selection might have acted on these vibrations, it is also necessary to examine how bees use
489 these vibrations for their particular functions. The biomechanical properties of a vibration might only
490 be part of what makes it effective. Other behaviours can increase the effectiveness of a particular
491 vibration by increasing the salience or memorability of a signal, such as when animals combine
492 multiple modalities into a signal (Rowe, 1999), or by modifying the effects of the vibrations, such
493 when tree crickets build acoustic baffles to amplify the volume of their mating calls (Mhatre et al.,
494 2017). During floral buzzing, bees do not simply applying vibrations like the artificial shakers used to
495 study pollen release. Instead, bees need to learn to handle flowers correctly, and work to get in
496 position before starting buzzing (Laverty, 1980; Macior, 1964; Russell et al., 2016). How bees handle
497 flowers, where they bite anthers, and how they position themselves as they vibrate, could all
498 influence how the high acceleration vibrations we recorded are applied to the flower and result in
499 pollen ejection. The next step for understanding why bumblebees, and other insects, produce the
500 vibrations they do, is to understand how other behaviours work alongside vibrations to serve their
501 function.

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509

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689 Tables

690 **Table 1.** Analysis of bee size (intertegular distance) and behavioural context on the properties of
 691 thoracic vibrations measured with a laser vibrometer. The parameter estimates and standard errors
 692 were calculated from a linear mixed effect model with bee identity as a random factor. *P*-values for
 693 each explanatory variable were calculated using a Type III analysis of variance with Satterthwaite’s
 694 estimation of degrees of freedom. Statistically significant values are in bold.

Response variable	Parameter	Estimate	SE	<i>P</i>-value
Peak Amplitude Velocity (mm/s)	Intercept (Buzz Type: Flight)	165.71	94.16	
	Intertegular distance	-24.63	21.72	0.27
	Buzz Type			< 0.001
	Defence	132.68	8.54	
	Floral	207.65	14.53.	
Fundamental Frequency (Hz)	Intercept (Buzz Type: Flight)	200.93	70.89	
	Intertegular distance	-14.53	16.36	0.38
	Buzz Type			< 0.001
	Defence	102.93	3.38	
	Floral	177.70	10.50	
	Buzz Type*Intertegular distance			0.002
Displacement (mm)	Intercept (Buzz Type: Flight)	0.24	0.11	
	Intertegular distance	-0.022	0.026	0.40
	Buzz Type			< 0.001
	Defence	0.11	0.011	
	Floral	0.13	0.017	
Acceleration (m/s²)	Intercept (Buzz Type: Flight)	358.32	199.45	
	Intertegular distance	-71.09	46.01	0.13
	Buzz Type			< 0.001
	Defence	248.57	16.82	
	Floral	479.57	30.57	

695

696

697 **Table 2.** Analysis of bee size (intertegular distance), plant species, and recording location on the
 698 properties of floral vibrations. Vibrations were recorded on *S. citrullifolium* and *S. rostratum*, both
 699 directly on the bee’s thorax using a laser vibrometer and on the flower using an accelerometer. The
 700 parameter estimates and standard errors were calculated from a linear mixed effect model with bee
 701 identity as a random factor. *P*-values for each explanatory variable were calculated using a Type III
 702 analysis of variance with Satterthwaite’s estimation of degrees of freedom. Statistically significant
 703 values are in bold.

704

Response variable	Variable	Estimate	SE	<i>P</i> -value
Peak Amplitude Velocity (mm/s)	Intercept (Plant: <i>S. citrullifolium</i> , Location: Bee)	312.06	74.43	
	Intertegular distance	-13.74	16.31	0.42
	Plant species: <i>S. rostratum</i>	22.22	12.95	0.11
	Location: Flower	-233.35	9.30	<0.001
Fundamental Frequency (Hz)	Intercept (Plant: <i>S. citrullifolium</i> , Location: Bee)	462.66	60.83	
	Intertegular distance	-33.54	13.36	0.027
	Plant species: <i>S. rostratum</i>	4.40	10.12	0.67
	Location: Flower	-0.20	2.07	0.92

705

706 **Table 3.** Effect of bee size (intertegular distance distance), flower species, and vibration method on
 707 the magnitude of King’s Coupling Factor. Vibrations were applied to *S. citrullifolium* and *S. rostratum*,
 708 either by the bee (bee) or by pressing a calibrated shaker against the flower (shaker). The parameter
 709 estimates and standard errors were calculated from a linear mixed effect model with bee identity as
 710 a random factor. P-values for each explanatory variable were calculated using a Type III analysis of
 711 variance with Satterthwaite’s method. Statistically significant values are in bold.

Response	Variable	Estimate	SE	P
Coupling Factor	Intercept (Flower: <i>S. citrullifolium</i> + Vibration source: Shaker)	14.26	5.92	
	Intertegular distance	-0.89	1.29	0.51
	Flower species			0.002
	<i>S. rostratum</i>	-4.04	1.03	
	Vibration source			0.72
	Bee	-0.32	0.91	

712

713

714 Figures

715

716 **Figure 1: Experimental set up for measuring bee vibrations.** For floral buzzes (A), vibrations were
 717 recorded simultaneously by a PDV-100 laser vibrometer focussed on a 2mm² reflective tag on the
 718 back of the thorax of the bee, and by a 0.2g accelerometer pinned to the calyx at the base of the
 719 flower. These measurements were sent to the compactRIO data acquisition unit (cRIO) which
 720 timestamped the data and exported them to a file. For defence and flight buzzes (bottom), bees
 721 were tethered on a platform using a nylon wire loop fed through a blunted needle. For defence
 722 buzzes (B), bees were gently squeezed on the abdomen using featherweight tweezers. To stimulate
 723 flight (C), the platform was dropped away triggering reflexive flight. In both cases, vibrations were
 724 recorded using a PDV-100 laser vibrometer positioned above the bee and aimed at a 2mm² on the
 725 back of the thorax. The vibrometer then send the data to the cRIO to be timestamped and exported.

726 **Figure 2. Oscillograms and frequency spectra of vibrations (buzzes) produced by bumblebees**
 727 **(*Bombus terrestris audax*) in three different behavioural contexts: Flight (A, B), defence (C, D), and**
 728 **buzz pollination (E, F).** Left-hand side panels (A, C, E) show buzzes in the time domain (oscillograms),
 729 while right-hand side panels show buzzes in the frequency domain (frequency spectra; B, D, F). The
 730 coloured region in the oscillogram show the section of the buzz used to generate the corresponding
 731 frequency spectrum. The first five harmonics (multiples of the fundamental frequency) are shown as
 732 vertical dashed lines in the frequency spectra.

733 **Figure 3. Differences in the properties of vibrations (buzzes) produced in different contexts (flight,**
 734 **defence, floral buzzes).** Vibrations differed in both peak velocity (A) and frequency (B), with floral
 735 buzzes exhibiting the highest velocity and highest frequency buzzes, and flight producing the lowest
 736 velocity and frequency vibrations. From these values we derived the magnitude of the vibrations, in
 737 terms of displacement of the thorax, (C) and the acceleration (D) produced during these vibrations.

738 Although there was no difference in the absolute magnitude of the vibrations produced during
 739 defence and floral buzzes, because the floral buzzes were faster and at higher frequency than the
 740 defence buzzes, floral buzzes showed much higher accelerations. Mean +- SE. N = 36 bees from 2
 741 colonies (16 for floral vibrations, 20 for defence and flight vibrations). Details of statistics in Table 1.

742 **Figure 4. Example of a floral vibration produced by *Bombus terrestris audax* while visiting a flower**
 743 **of *Solanum citrullifolium* as recorded directly from the bee (A, B) and on the flower (C, D).** The
 744 magnitude of the vibration, measured as peak velocity amplitude, is much higher when measured
 745 directly on the bee's thorax with a laser vibrometer (A), than when measured using an
 746 accelerometer attached to the base of the flower (C). In contrast, the fundamental frequency of the
 747 buzz produced during floral visitation is the same (355 Hz) whether is measured in the bee's thorax
 748 (B) or on the base of the flower (D). The coloured section in the oscillograms shown in A and C
 749 represent the section of the buzz used to calculate the frequency spectra shown in B and D. The
 750 dashed lines in panels B and D represent the first five harmonics of the fundamental frequency.

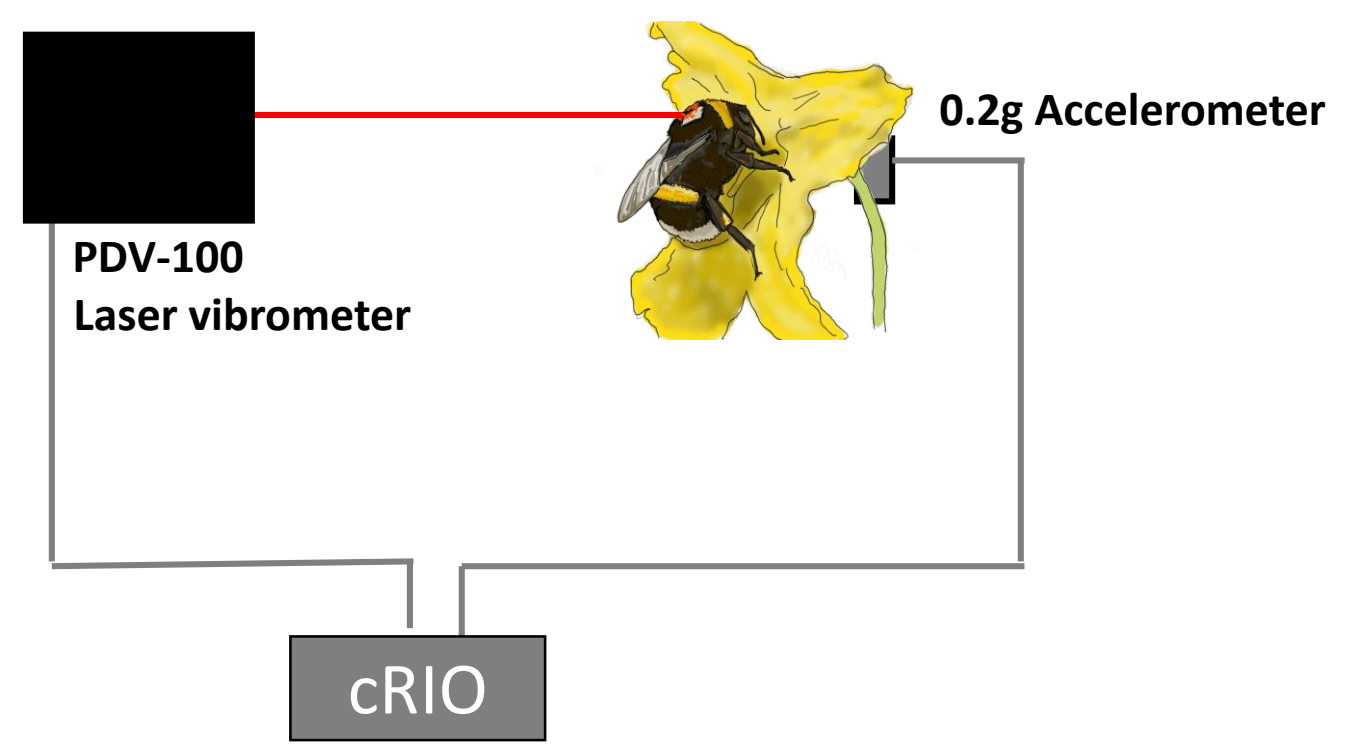
751 **Figure 5. Peak amplitude velocity (A) and fundamental frequency (B) of floral buzzes of *Bombus***
 752 ***terrestris audax* on buzz-pollinated flowers of *Solanum rostratum* (closed symbols) and *S.***
 753 ***citrullifolium* (open symbols).** Floral buzzes were recorded directly from the bee's thorax using a
 754 laser vibrometer (green symbols) or on the flower using an accelerometer attached to the calyx
 755 (magenta symbols). Vibrations recorded on the flower had significantly lower peak velocities but
 756 similar fundamental frequencies as those measured in the bee. (C) Relationship between bee size
 757 (intertegular distance) and the fundamental frequency of floral buzzes. Each symbol in (C) represents
 758 the average frequency from multiple buzzes produced by an individual bee. Mean +- SE for A & B. N
 759 = 16 bees from 2 colonies (8 on *S. rostratum*, 8 on *S. citrullifolium*). Details of statistics in Table 2.

760 **Figure 6. Comparison of the ratio of the magnitude of the input vibration to the magnitude of the**
 761 **vibration measured at the sensor (King's coupling factor) on two buzz-pollinated species of**
 762 ***Solanum*.** Coupling factors were estimated using either natural bee vibrations (*bee*) or synthetic

Properties of vibrations in bumblebees

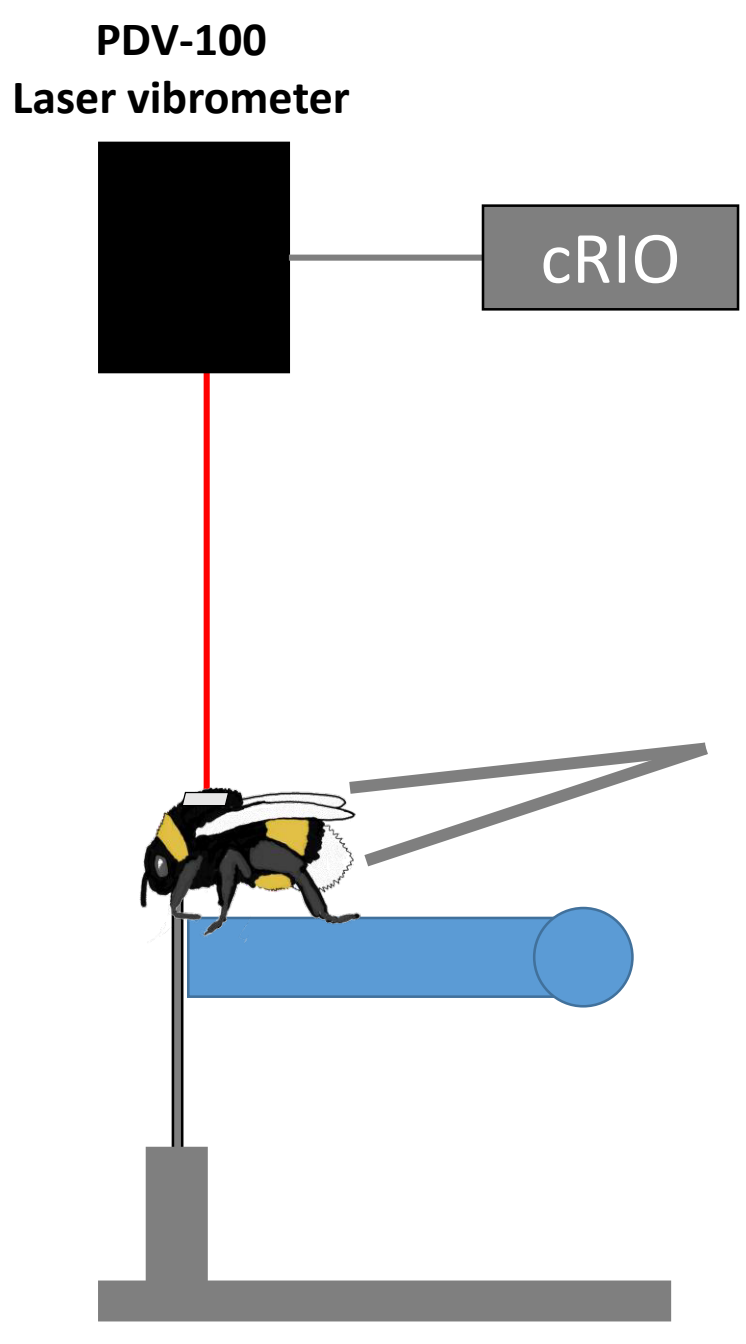
763 vibrations produced with a calibrated mechanical shaker (*shaker*) as the input vibration. The
764 calibrated shaker produced a vibration of fixed properties (frequency = 159.2 Hz, RMS velocity =
765 9.8mm/s). The magnitude of the vibration produced by the bee was measured using a laser
766 vibrometer on the bee's thorax. The vibration transmitted to the sensor on the flower was measured
767 at the calyx using an accelerometer. Plant species consistently differ in their coupling factor with *S.*
768 *rostratum* having lower values than *S. citrullifolium* (A), irrespective of whether it is calculated using
769 bee or calibrated shaker vibrations (B). Mean +- SE. A: N = 16 bees from 2 colonies (8 on *S.*
770 *rostratum*, 8 on *S. citrullifolium*), and 13 manual vibrations of flower (6 on *S. rostratum*, 6 on *S.*
771 *citrullifolium*). B: N = 13 bees who had matching manual vibrations of their flower (6 on *S.*
772 *rostratum*, 7 on *S. citrullifolium*). Details of statistics in Table 3

Floral buzzes



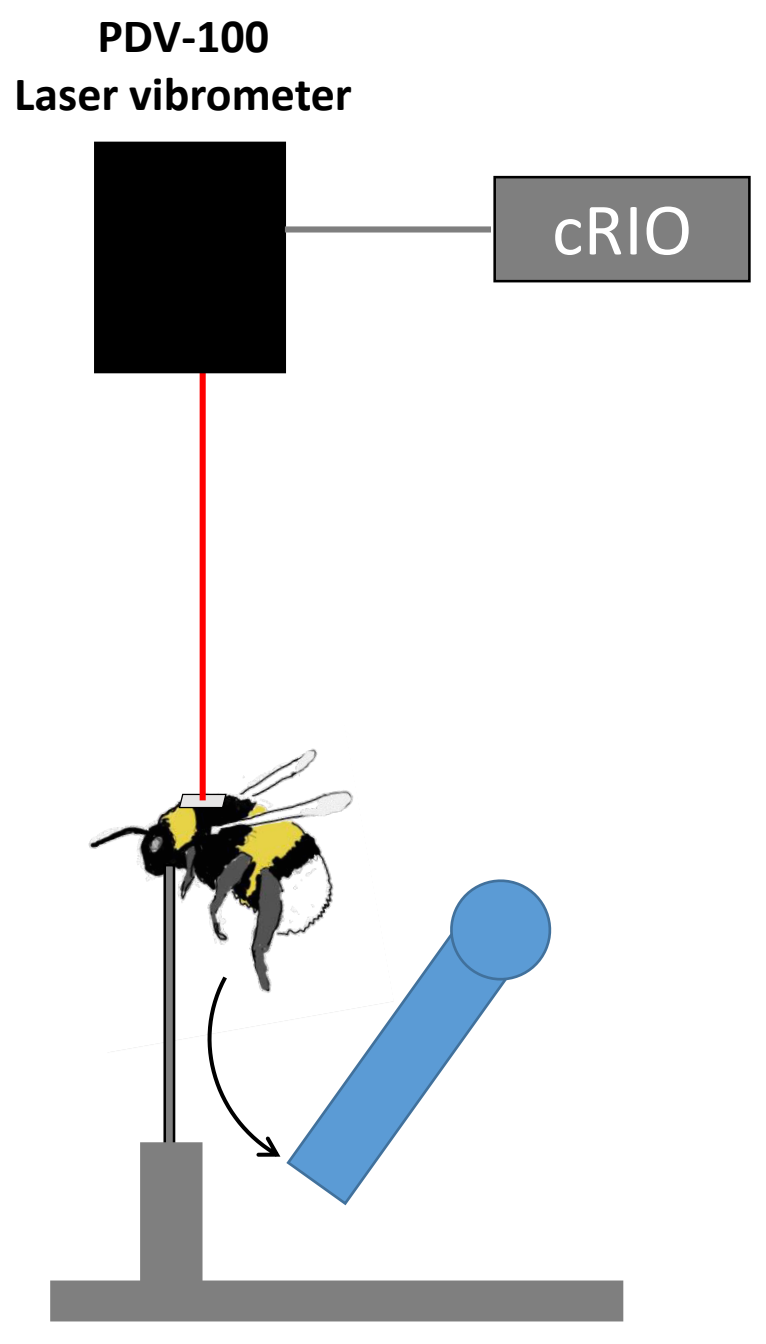
A

Defence buzzes



B

Flight buzzes



C

Figure 1

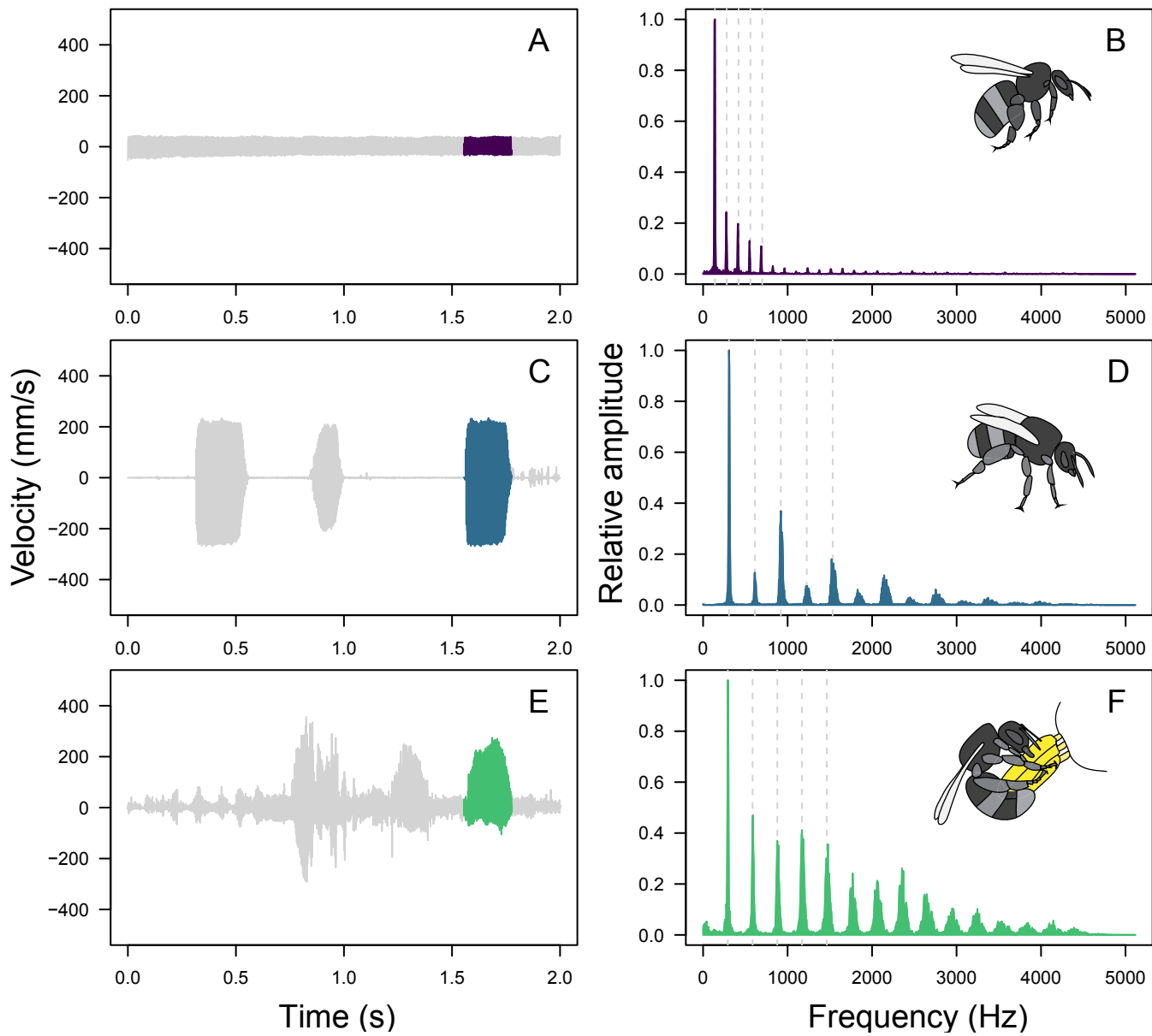
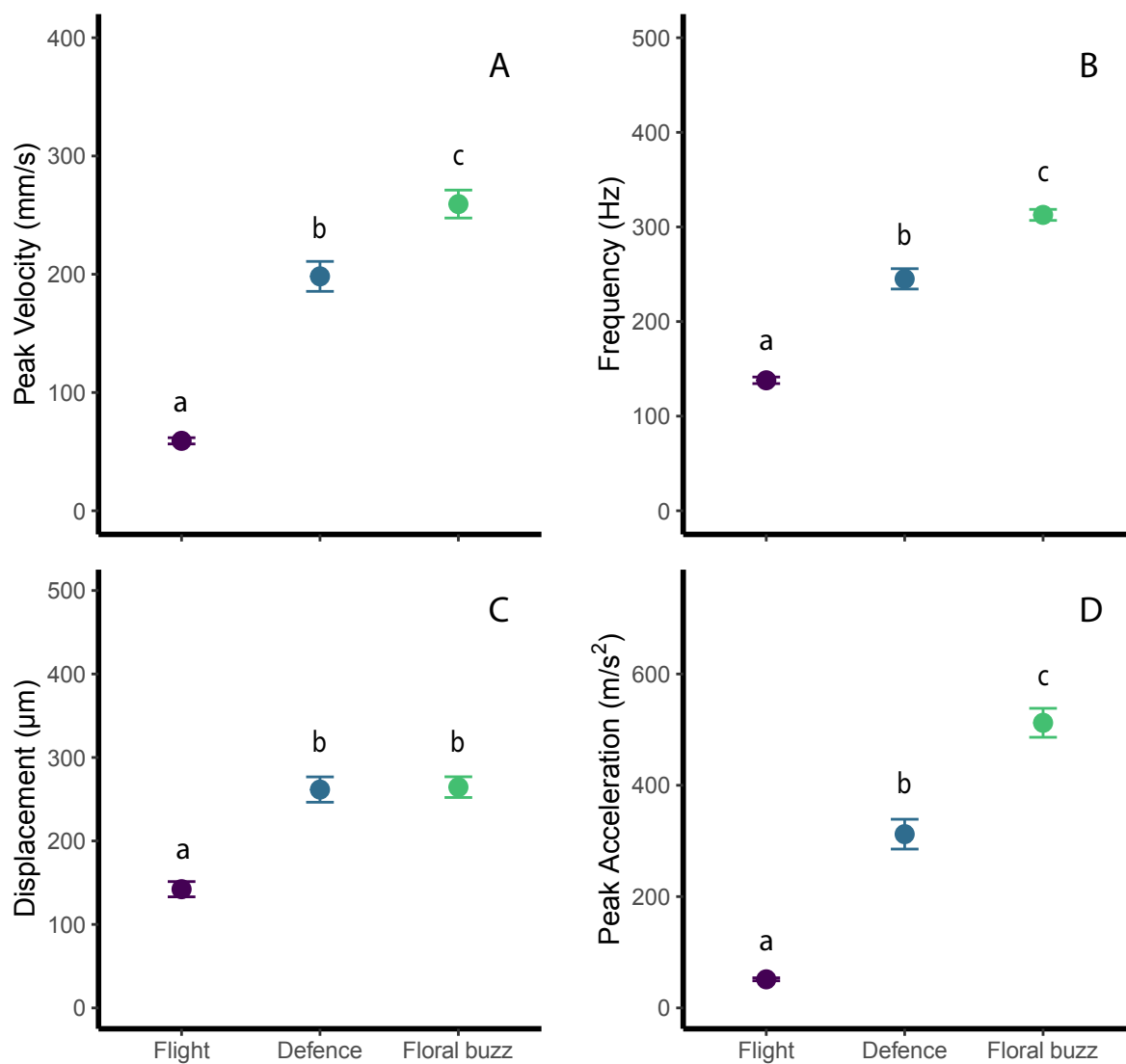


Figure 2



Buzz Type

Figure 3

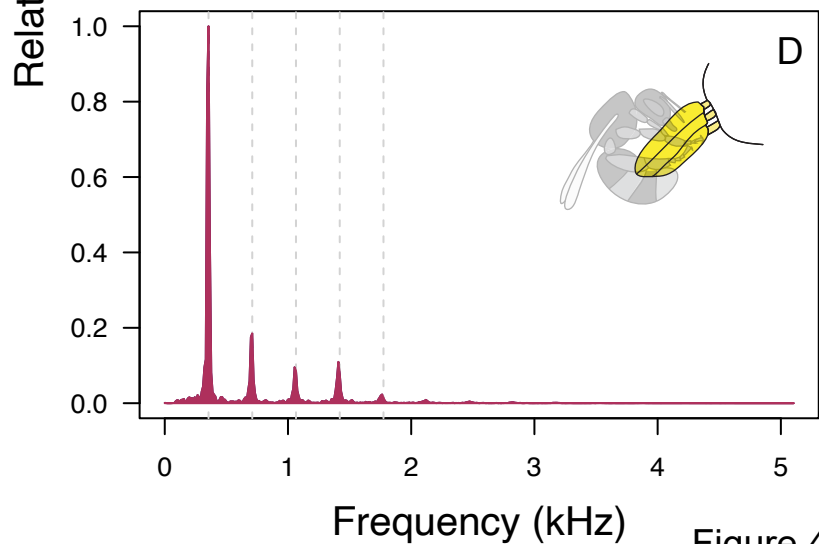
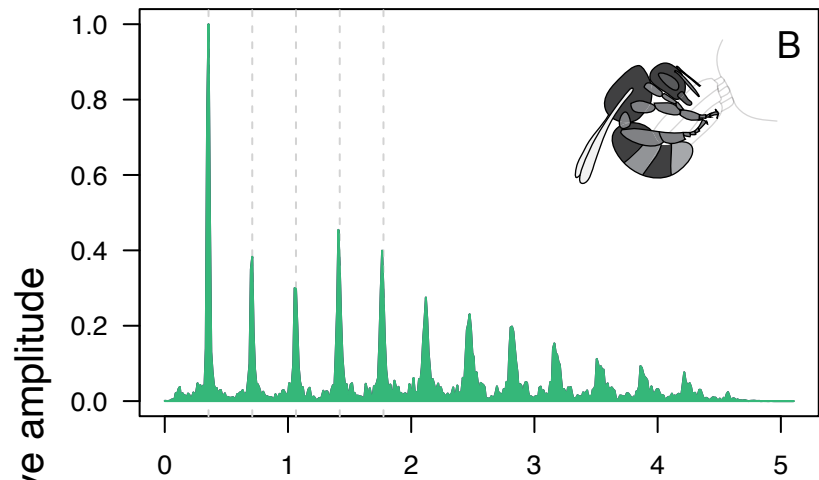
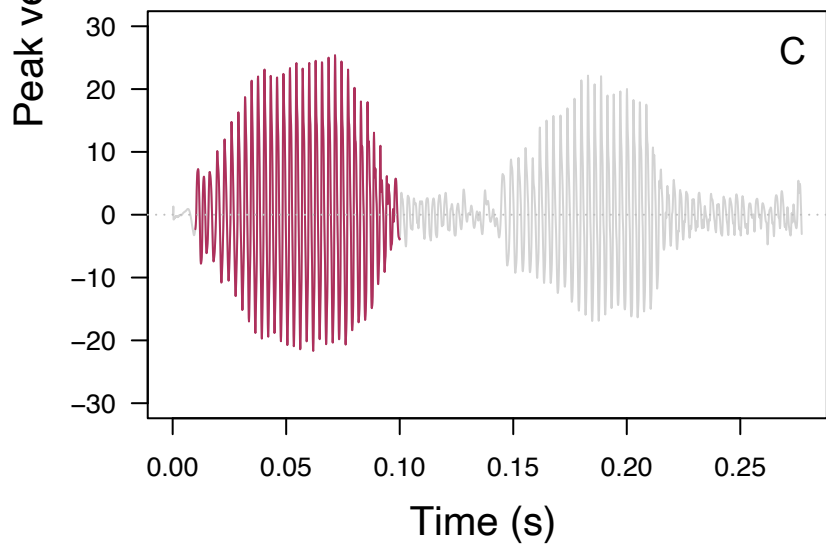
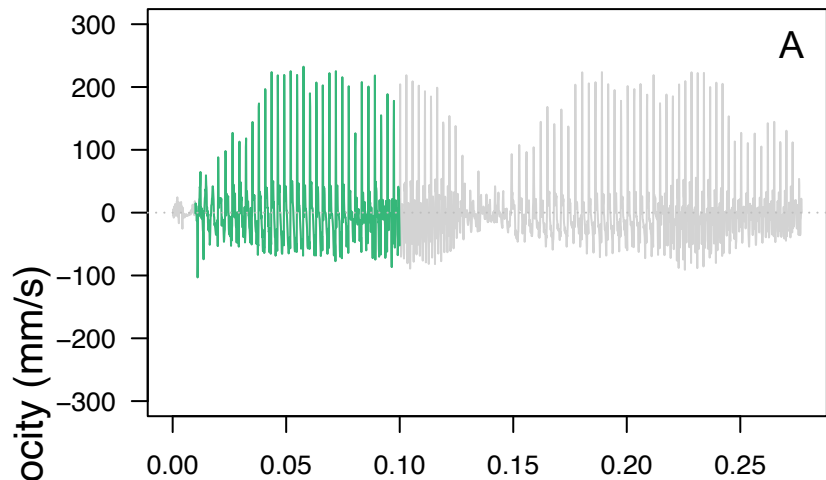
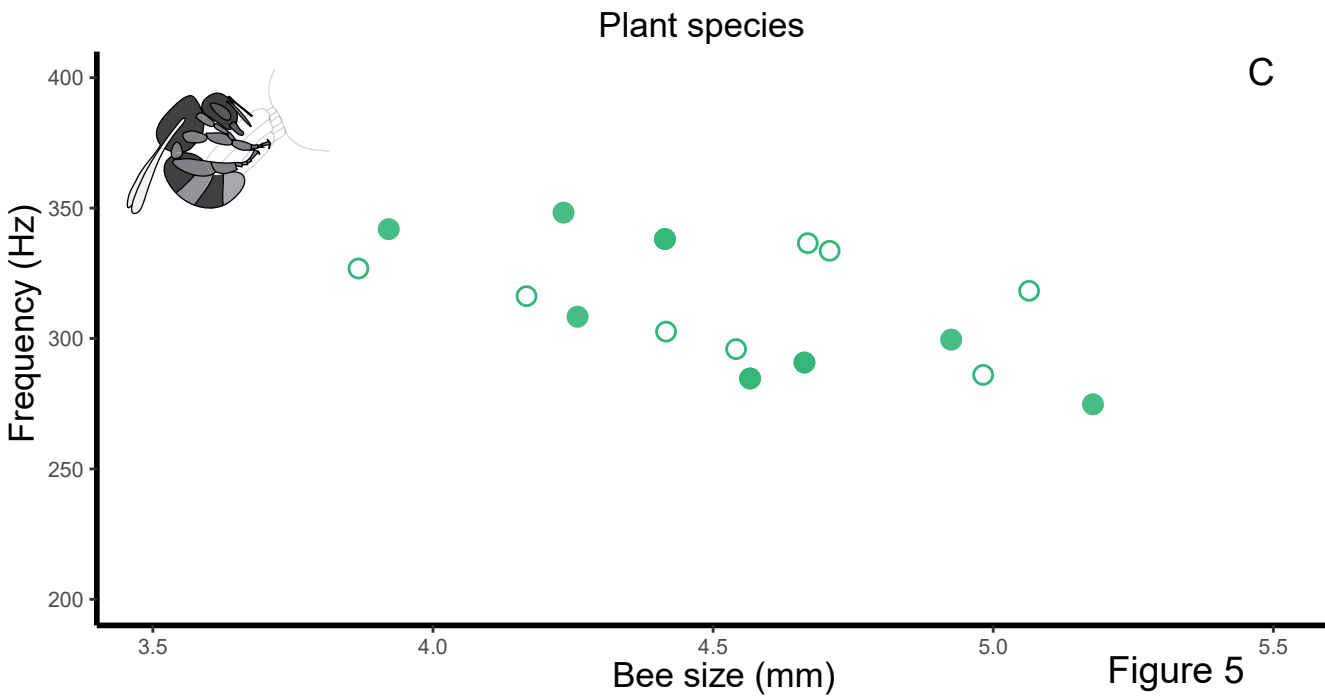
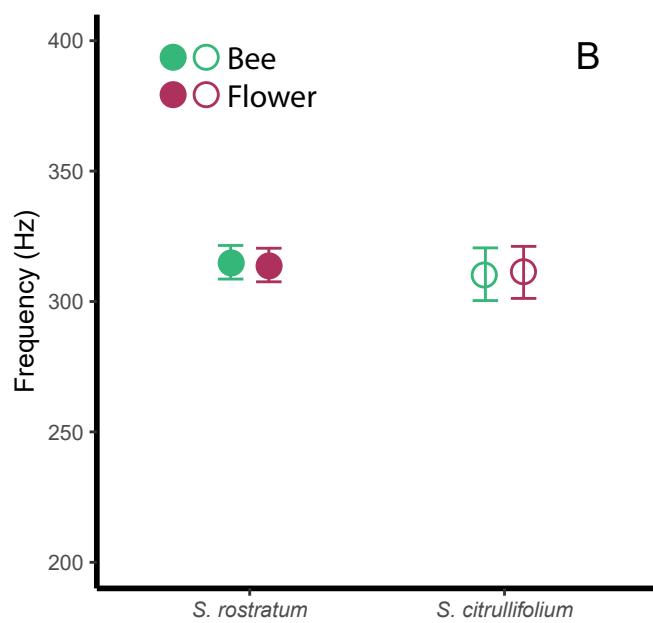
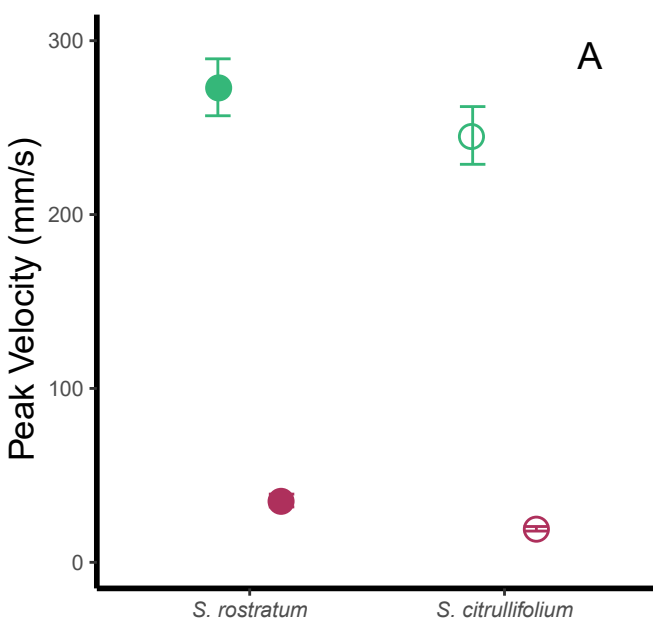
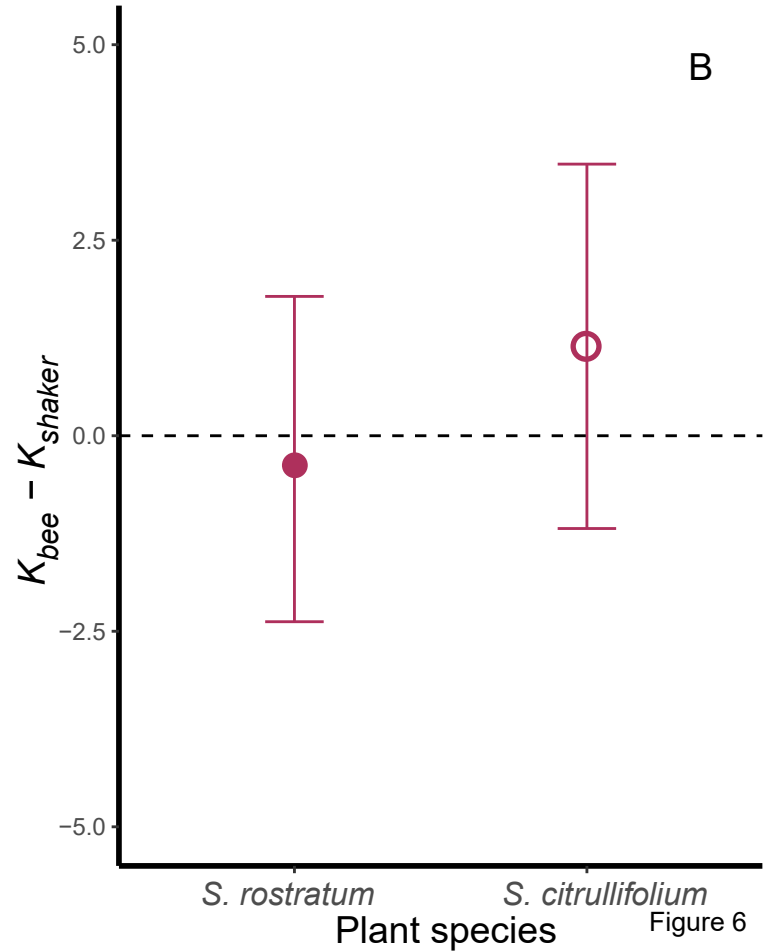
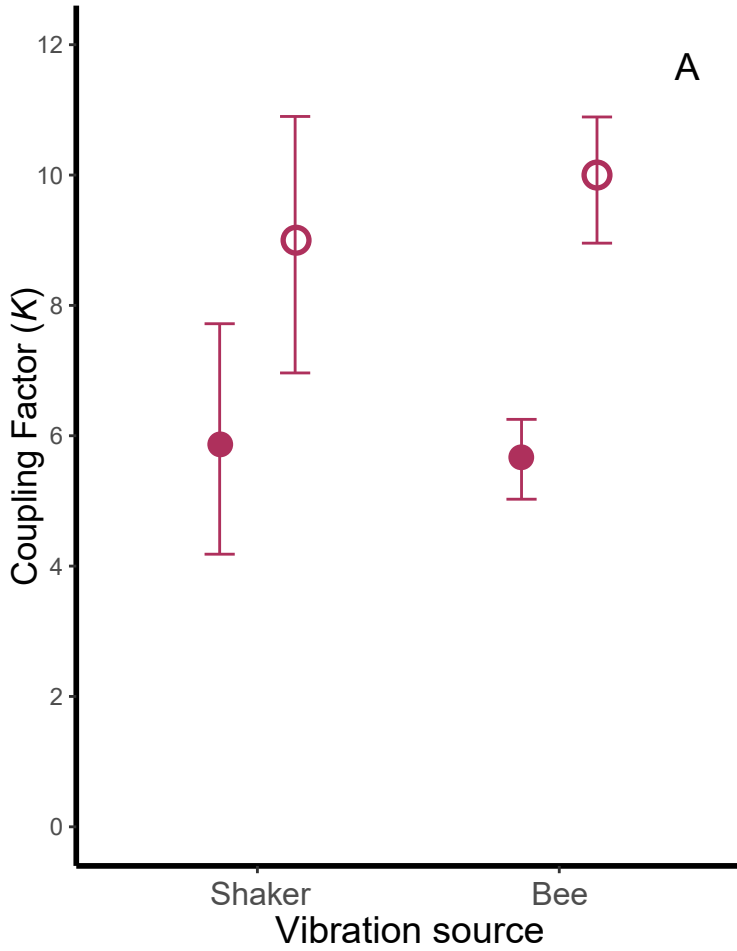
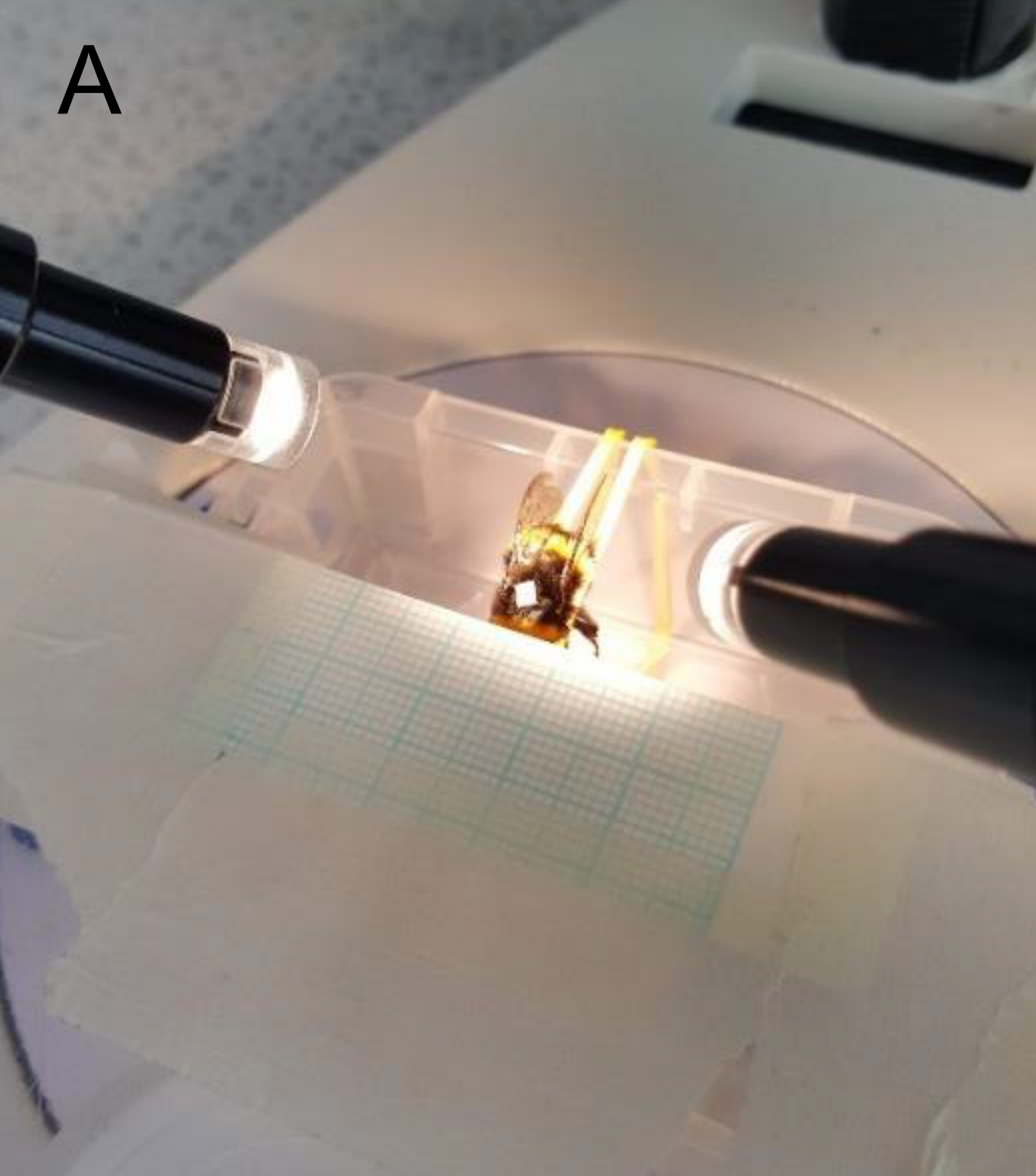


Figure 4





A



B

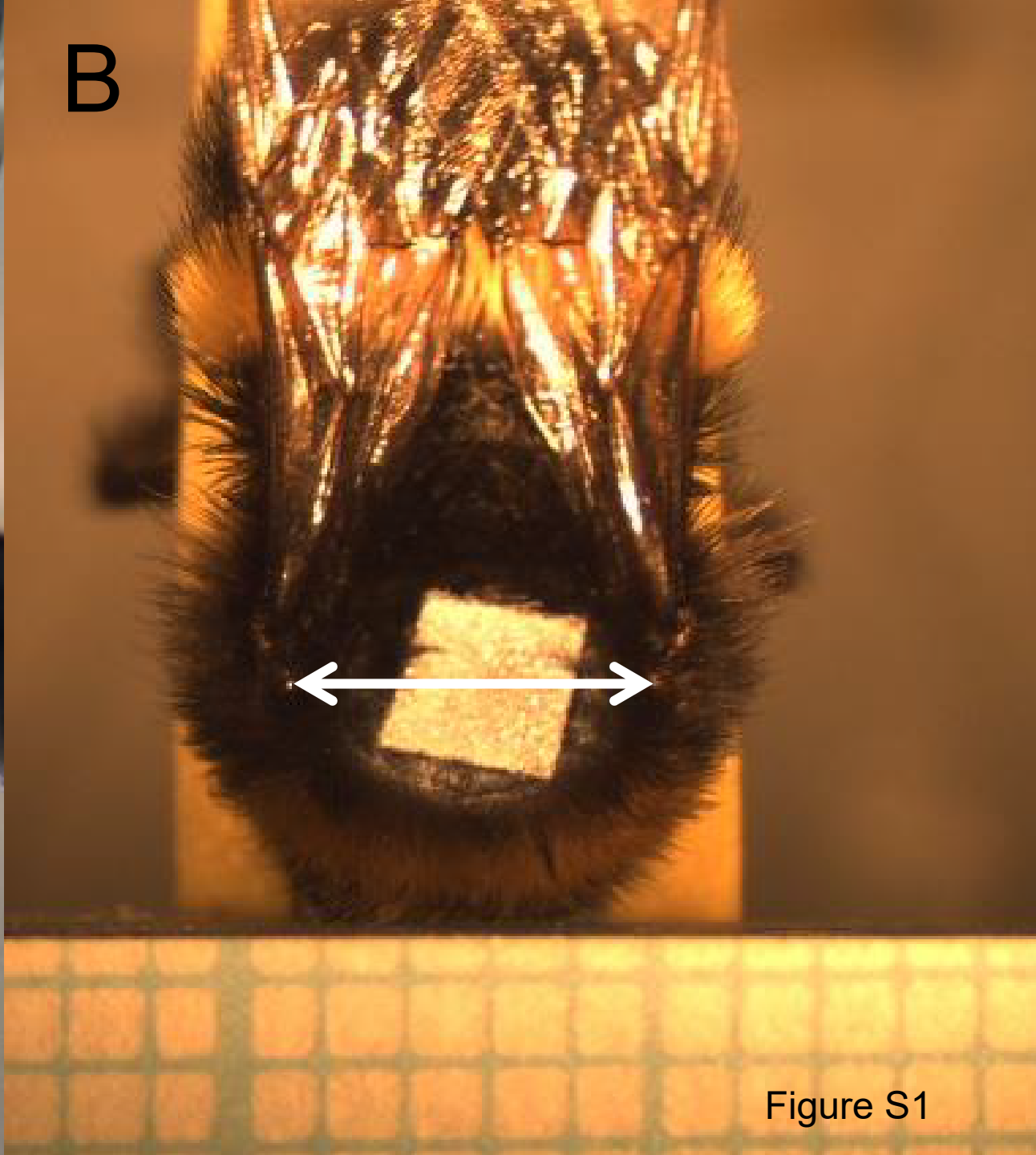


Figure S1

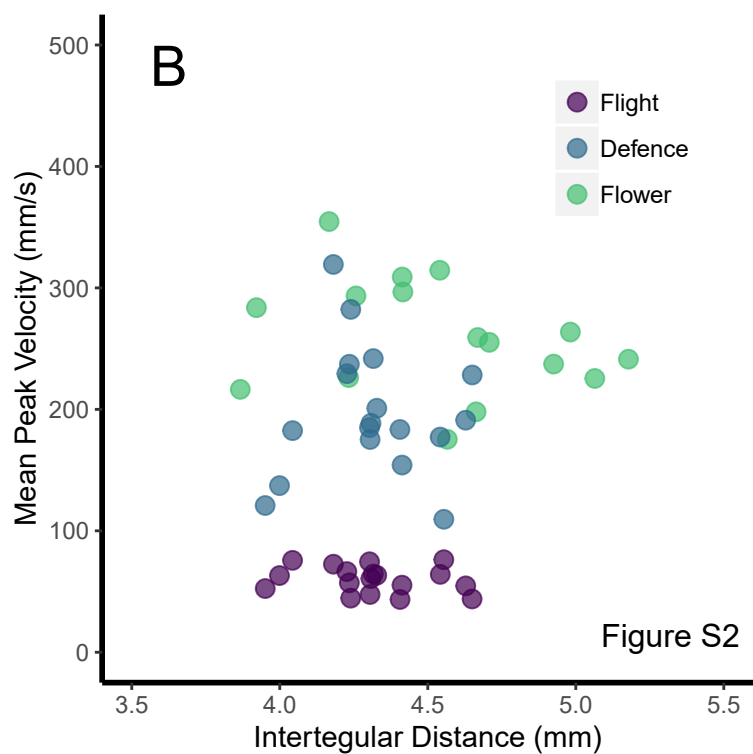
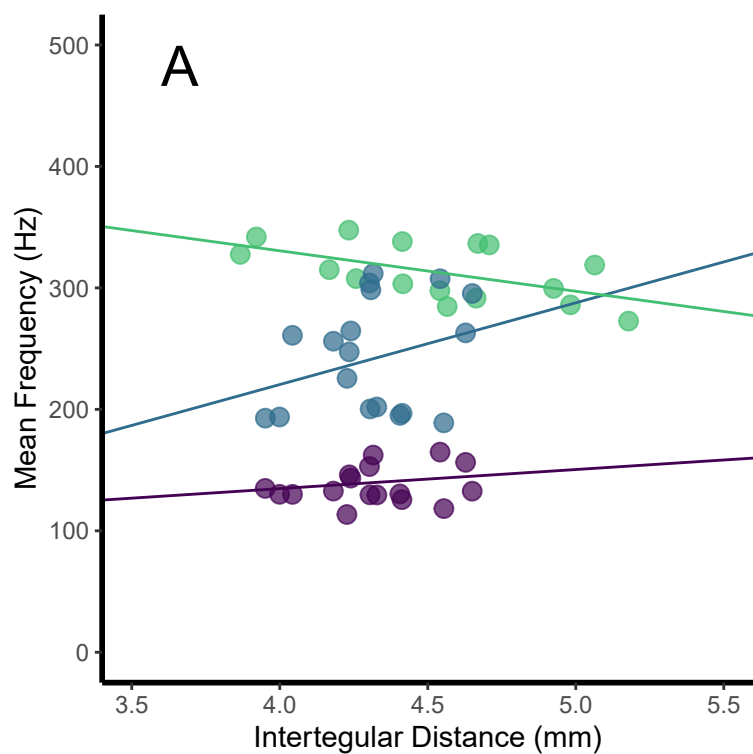


Figure S2