1 **Variation in natural frequency of stamens in six morphologically diverse, buzz-**

2 **pollinated, heterantherous *Solanum,* and its relationship to bee vibrations**

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14 **Running head:** NATURAL FREQUENCIES IN BUZZ POLLINATION

15   ABSTRACT

16   During buzz pollination, bees use vibrations to remove pollen from flowers. Vibrations at the

17   natural frequency of pollen-carrying stamens are amplified through resonance, resulting in

18   higher-amplitude vibrations. Because pollen release depends on vibration amplitude, bees

19   could increase pollen removal by vibrating at the natural frequency of stamens. Yet, few

20   studies have characterized the natural frequencies of stamens and compared them to the

21   frequencies of buzz-pollinating bees. We use laser Doppler vibrometry to characterise natural

22   frequencies of stamens of six morphologically diverse, buzz-pollinated, heterantherous

23   *Solanum,* and compare the frequency of bumblebee buzzes produced on two *Solanum* species

24   with different natural frequencies. We found that stamen morphology and plant identity

25   explain variation in their natural frequency. The natural frequencies of the studied *Solanum*

26   stamens fall between 45-295 Hz with 5/6 species being <190 Hz, which only partly overlaps

27   floral vibrations of buzz-pollinating bees. We show that captive bumblebees produce

28   vibrations at a frequency of 345 Hz, and do not change their floral vibrations to match the

29   natural frequency of the visited flowers. Our results suggest that pollen release induced by

30   vibrating stamens at their natural frequencies might only play a role in a subset of buzz

31   pollination interactions.

32

33   KEYWORDS

34   Biomechanics – *Bombus terrestris* – buffalo-bur –buzz pollination – flower diversity –

35   nightshade – resonance – *Solanum citrullifolium* – *Solanum rostratum* – Solanaceae

INTRODUCTION

37 More than half of all bee species evolved the ability to vibrate to extract pollen from flowers,

38 giving rise to the syndrome of buzz pollination (Buchmann, 1983; Cardinal, Buchmann, &

39 Russell, 2018; Vallejo-Marín, 2019). Most buzz-pollinated flowers present evolutionarily

40 derived morphologies in which pollen locked inside stamens is released through small pores

41 (poricidal stamens) (Buchmann, 1983). While buzzing flowers, bees hold stamens using their

42 mandibles and legs and activate their thoracic muscles (De Luca & Vallejo-Marín, 2013,

43 Figure 1A). Pollen release from poricidal stamens is a function of the vibration

44 characteristics, mainly its amplitude (Harder & Barclay, 1994; King & Buchmann, 1996; De

45 Luca *et al.*, 2013; Kemp & Vallejo-Marín, 2020; Rosi-Denadai *et al.*, 2020). The amplitude

46 of a floral vibration depends on the characteristics of the bee (King & Buchmann, 2003), the

47 coupling between bee and flower (King, 1993; Arroyo-Correa, Beattie, & Vallejo-Marín,

48 2019), and the vibrational properties of the stamen (anther and filament) (Buchmann &

49 Hurley, 1978; Mortimer, 2017; Vallejo-Marín, 2019; Brito *et al.*, 2020).

50 One vibrational property of solid structures, including stamens, is the natural

51 frequency. Natural frequencies are the frequencies at which objects vibrate when disturbed,

52 and are given by their mass, shape and material properties, such as rigidity or stiffness

53 (Volterra & Zachmanoglou, 1965; Niklas, 1992). When a structure is vibrated at its natural

54 frequency it resonates, causing higher amplitude vibrations. The first natural frequency is the

55 lowest frequency at which an object resonates. Complex systems can have more than one

56 natural frequency (Volterra & Zachmanoglou, 1965; Niklas, 1992). Stamens may behave

57 analogously to a cantilever beam (King & Buchmann, 1995), a structure fixed at one end and

58 free at the other, which has multiple normal modes (Fletcher 1992). The first normal mode, or

59 natural frequency, of a cantilever beam-like structure should correspond to the highest

60 achievable amplitude at resonance (Volterra & Zachmanoglou, 1965). In principle, if the

61 vibrations applied by bees occurred at the natural frequency of stamens, vibration amplitude

62 would increase through resonance, resulting in higher pollen removal (King & Buchmann,

63 1996; Timerman & Barrett, 2019).

64   The relevance of stamen natural frequencies for pollen release is linked to the type of

65 vibrations that bees can produce. In bees, the fundamental frequency of floral vibrations is

66 distinct (higher) than vibrations produced in other behaviours such as flight or defence

67 (Pritchard & Vallejo-Marín, 2020). Extensive previous work on the spectral properties of

68 buzz pollination has established that the fundamental frequency of bee vibrations on flowers

69 varies across bee species and ranges from approximately 100 to 400 Hz with significant

70 variation within and among bee taxa (De Luca & Vallejo-Marín 2013; Corbet & Huang,

71 2014; Switzer & Combes, 2017; De Luca *et al.*, 2019; Pritchard & Vallejo-Marín, 2020;

72 Rosi-Denadai *et al.*, 2020). The cause of this variation in the frequency of floral vibrations

73 remains unknown. Unlike the frequency of flight vibrations, which varies negatively with

74 individual size, the variation in fundamental frequency of floral vibrations across bee species

75 is not strongly associated with size across species (De Luca *et al.*, 2019). Bees might use

76 vibrations of different frequency when visiting different species of buzz-pollinated flowers

77 (Corbet & Huang, 2014; Switzer & Combes, 2017). However, experimental studies with

78 captive bumblebees have not found large changes in buzz frequency when the same bee

79 species visits different flower species (Arroyo-Correa *et al.*, 2019) or when trained in

80 artificial flowers that release pollen at different specific frequencies (Switzer *et al.*, 2019).

81 Regardless of whether bees can adjust their vibration frequency to match the flowers they

82 visit, it is unclear whether the frequencies of bees' floral vibrations overlap the natural

83 frequencies of stamens.

84   In contrast to the numerous studies on the spectral properties of buzz pollinating bees,

85 to date, little is known about the natural frequencies of stamens of buzz-pollinated plants. A

86 pioneer study by King and Buchmann (1996) found that the natural frequency of stamens of

87 *Solanum laciniatum* Aiton was significantly lower (124 Hz) than the fundamental frequencies

88 of bees buzzing these flowers (316 Hz). Other studies on natural frequencies of flowers have

89 focused on wind-pollinated plants, in which vibrations induced by air flow lead to pollen

90 ejection (Timerman *et al.*, 2014; Timerman & Barrett, 2018, 2019). Clearly, further work is

91 needed to document the natural frequencies of other buzz-pollinated flowers and compare

92 them to the types of vibrations produced by bees.

93   Here, we exploit natural variation both between and within plant species, to

94 investigate the natural frequency of buzz-pollinated flowers. We use an unusual group of

95 *Solanum* L. (Solanaceae) species that captures repeated independent transitions in flower and

96 stamen morphology (Vallejo-Marín *et al.*, 2014). Unlike most *Solanum* species (Särkinen *et*

97 *al.*, 2013)*,* species in *Solanum* section *Androceras* Whalen are heterantherous, bearing two

98 sets of stamens with different morphologies specialised on either attracting and rewarding

99 pollinators (feeding stamens) or fertilisation (pollinating stamens) (Müller, 1881; Vallejo-

100 Marín *et al.*, 2009). The flowers of *Solanum* section *Androceras* studied here have four

101 smaller stamens located towards the centre of the flower (feeding stamens), and a single

102 stamen, usually larger, curved, and located away from the centre of the corolla (pollinating

103 stamen)(Whalen, 1978, 1979; Vallejo-Marín *et al.*, 2009; Figure 1A). We study three pairs of

104 closely related taxa in which one member is large-flowered and highly heterantherous, with

105 larger more conspicuous pollinating anthers, while the other is small-flowered and less

106 heterantherous, with smaller less conspicuous pollinating anthers (Whalen, 1978, 1979; Stern,

107 Weese, & Bohs, 2010; Vallejo-Marín *et al.*, 2014). This combination of within-flower

108 variation in stamen morphology represented by stamens with distinct morphologies within

109 the same flower in some species and phylogenetically independent floral morphology

110 transitions provides a system to investigate variation in natural frequencies in buzz-pollinated

flowers. Our study addresses two questions: (1) To what extent do stamens with different

morphologies have different natural frequencies? (2) Do bumblebees dynamically adjust the

frequency of their vibrations while visiting flowers that differ in the natural frequency of their

stamens?

MATERIAL AND METHODS

PLANT STUDY SYSTEM

We studied six taxa of *Solanum* section *Androceras* (Solanaceae) native to Mexico and the

southern USA that represent three pairs of closely related or sister taxa in each of the three

series in *Androceras* (see Table S1 for accession information): *Solanum fructu-tecto* Cav. and

Solanum rostratum Dunal of series *Androceras*, *Solanum citrullifolium* A.Braun and *Solanum*

heterodoxum Dunal of series *Violaceiflorum*, and *Solanum grayi* var. *grandiflorum* Whalen

and *Solanum grayi* var. *grayi* Whalen of series *Pacificum* (Whalen, 1979; Stern, Weese, &

Bohs, 2010). As in other wild *Solanum,* the pollinators of *Solanum* section *Androceras*

include buzz-pollinating bees of a varied range of sizes including bumblebees, *Bombus*

Latreille 1802 spp., which have been observed in *S. rostratum*, and *S. angustifolium* Mill.*;*

and other medium-sized bees (*Centris* Fabricius 1804 sp.) observed in *Solanum grayi*

grandiflorum and *S. lumholtzianum* Barlett (MVM *personal observation*). Published studies

of the pollination ecology of *Solanum* section *Androceras* are largely restricted to the widely

distributed *Solanum rostratum*. This species is pollinated by diverse buzz-pollinating visitors,

including small bees such as *Augochloropsis* Cockerell 1897, *Exomalopsis* Spinola 1853*,*

Lasioglossum Curtis 1833, and medium to large-sized bees such as *Bombus, Centris*,

Thygater Holmberg 1884, and *Xylocopa* Latreille 1802 (Bowers, 1975; Solís-Montero,

Vergara, & Vallejo-Marín, 2015; Solís-Montero *et al.*, 2018). In its introduced range in

135 China, *S. rostratum* is also visited by similar bees including *Halictus* Latreille 1804, *Bombus*

136 and *Xylocopa* (Zhang & Lou, 2015).

137   For this study, plants were germinated from seeds previously collected in the field (all

138 taxa except *S. citrullifolium*) or obtained from the Solanaceae collection previously kept at

139 the Radboud Botanic Gardens (*S. citrullifolium*; see Table S1 for source and accession

140 numbers). Seed germination and plant growth was carried out at the University of Stirling

141 plant growth facilities. Briefly, seeds were germinated following a 24h treatment with 1000

142 ppm gibberellic acid (GA3; Sigma-Aldrich, Dorset, UK) aqueous solution. Germinated

143 seedlings were transplanted after 2-3 weeks to 1.5L pots containing a mix of All Purpose

144 Growing Medium and Perlite Standard (4:1; William Sinclair Horticulture PLC, Lincoln,

145 UK), and fertilised weekly with Tomorite Concentrated Tomato Food (Levington, Surrey,

146 UK). Supplemental light was provided by compact fluorescent lamps for 16h per day and

147 supplemental heating was provided to maintain minimum temperatures at 16ºC and 25ºC

148 (night and day, respectively). A subset of plants was transplanted 2-3 weeks later to a large

149 bench with the same soil mix (approx. 5m x 1m x 70cm) with plants spaced 60cm apart, to

150 encourage flowering. The large benches had supplemental heating but not supplemental light.

151

152   NATURAL FREQUENCY OF STAMENS

153 Vibration measurements were done in a laboratory with controlled temperature and humidity

154 (21°C; 60% RH). Flowers for the experiment were collected in the morning of each

155 measurement day, from 8:00hs to 9:00hs, by cutting entire inflorescences and placing the

156 inflorescence stalk in water. We only used unvisited flowers that opened on the same day of

157 the measurements. In these species, poricidal anthers are dehiscent upon anthesis. We used a

158 single stamen cut at the base of the filament where it connects with the receptacle and

159 measured two stamens from each flower, one feeding and one pollinating. Stamens cut from

160   flowers were kept inside a plastic container lined with humid paper towels until ready to be

161   measured. Measurements from cut stamens were taken as quickly as possible to avoid

162   desiccation and potential changes in the stamens' material properties.

163   To measure natural frequencies, we estimated frequency spectra of stamens exposed

164   to broad-band white noise (King, 1993; King & Buchmann, 1995, 1996, 2003). Single

165   stamens were exposed to white noise vibrations (a randomly generated mix of frequencies

166   between 20 and 20,000 Hz) generated in *Audacity* (version 2.4.1, Audacity Team, 2019),

167   using a linear power amplifier (LDS-LPA100, Brüel & Kjær, Nærum, Denmark) and a

168   permanent magnetic shaker platform (LDV210, Brüel & Kjær). Each stamen was glued

169   (Loctite Ultra Gel Control, Henkel, Hemel Hempstead, UK) by its filament base to a rigid

170   platform at the top of the shaker (Figure 1B). As even small changes in mass might affect

171   their dynamic properties, we applied very low accelerations which were not sufficient to

172   remove pollen from flowers and ensured that the mass of the flower remained constant

173   throughout each measurement.

174   We measured the vibration response of stamens using a laser Doppler vibrometer,

175   which uses the Doppler effect of a laser beam reflected on a target surface to estimate

176   vibrational properties, without the need of physical contact between the measuring equipment

177   and the target. We used a PDV-100 laser Doppler vibrometer (Polytec, Waldbronn,

178   Germany) set to 500 mm s^{-1} sensitivity, a low-pass filter of 5 kHz, and no high-pass filter. We

179   focused the laser beam as close to the apical end of the stamen as possible at an axis

180   perpendicular to the stamen length, parallel to the main axis of displacement of the shaker

181   platform (Figure 1B). An accelerometer (0.8 grams, 352A24, PCB Piezotronics, Depew, NY)

182   was attached to the shaker to record reference measurements. Both the laser vibrometer

183   (recorded in acceleration units) and the accelerometer signals were simultaneously acquired

184   using VibSoft-20 (Polytec) at a sampling rate of 12,000 samples per second, using a 20-5,000

185   Hz bandpass filter, and recorded for 1.28 s (15,360 samples; resolution 0.781 Hz). We

186   obtained the frequency spectra in the range from 20 to 2,500 Hz using a Fast Fourier

187   transform (FFT; 6,375 lines with a Hamming window) using VibSoft-20 and calculated the

188   average frequency spectrum of 10 replicate measurements for each stamen.

189   To estimate the first natural frequency (hereafter natural frequency), we visually

190   identified the first (lowest frequency) peak in the frequency spectrum (20-600 Hz range) and

191   obtained its associated frequency. This value corresponds to the first natural frequency (King

192   & Buchmann, 1996; Timerman *et al.*, 2014). The first natural frequency in a cantilever beam

193   is expected to be associated with the highest resonance amplitude (Volterra &

194   Zachmanoglou, 1965). Natural frequencies were assessed in an average of 10 flowers per

195   taxon (range 5-11, n = 54 flowers) from 2-8 individuals per taxon (two for *S. fructu-tecto*;

196   average of 1.46 flowers per individual, n = 41 individuals; Table S1).

197   We measured eleven stamen and floral traits and calculated two others from those,

198   totalling 13 floral traits for the same 54 flowers used to calculate natural frequencies to

199   establish correlations among traits that could influence natural frequencies. The measured

200   traits were flower mass, corolla height, corolla width, stamen length, anther length, anther

201   major diameter, anther minor diameter, filament major diameter, filament minor diameter,

202   stamen mass, anther mass. These traits were measured separately for pollinating and feeding

203   stamens, and filament lengths and filament masses were calculated from the above

204   measurements.

205

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

208   We compared the floral vibrations produced by captive bumblebees while visiting two plant

209   species with contrasting floral morphologies: *S. citrullifolium* and *S. heterodoxum*. These two

210   plant species are closely related but differ strongly in their floral morphology as well as in the

211 first natural frequencies of their stamens (see Results). *S. citrullifolium* has relatively large

212 flowers, the two anther types are well differentiated with the single pollinating anther being

213 larger in size, S-shaped, and coloured violet, in contrast to the four smaller, straight-shaped,

214 yellow-coloured feeding anthers (Vallejo-Marín *et al.*, 2014). The size of the flowers of *S.*

215 *citrullifolium* is similar to or larger than the bumblebee-pollinated flowers of *S. rostratum* and

216 is likely visited by similarly sized-pollinators although we have not been able to locate

217 published accounts of its pollination ecology. In contrast, the flowers of *S. heterodoxum* are

218 much smaller, with smaller anthers and less differentiated anther types, being almost

219 isoantherous (Vallejo-Marín *et al.*, 2014), and sets abundant fruits in the absence of

220 pollinators (authors' observation). The small size of its anthers might make its flowers

221 difficult to manipulate by large-bodied bumblebees.

222   We used one colony of *Bombus terrestris subsp. audax* Harris 1776 (Biobest,

223 Westerlo, Belgium; hereafter *B. terrestris*). We provided the colony with sugar solution

224 (Biobest) *ad libitum*. The colony was attached to a flight arena (122 x 100 x 37 cm),

225 illuminated with an LED light panel (59.5×59.5 cm, 48 W Daylight; Opus Lighting

226 Technology, Birmingham, UK) and maintained on a 12:12 h supplemental dark:light cycle.

227 Room temperature was 22-23°C and humidity was 50-60% RH. Although *B. terrestris* is

228 native to Europe and hence not a natural pollinator of *Solanum* Section *Androceras,* we

229 considered this bee species as a useful model to study bee vibrations on buzz-pollinated

230 flowers as the *Solanum* taxa studied are pollinated by buzzing bees of similar size, including

231 bumblebees, in their native range (Solís-Montero *et al.*, 2015).

232   We placed a single flower of either *S. citrullifolium* or *S. heterodoxum* in the flight cage,

233 allowing a bee to forage freely for approximately 10 minutes (visitation bout). We recorded up

234 to three minutes of floral buzzes using a digital audio recorder with two unidirectional

235 condenser microphones (Zoom H4n Pro Handy, Zoom North America, Hauppauge, NY)

236 placed always at 10 cm from the flower, sampling the audible component of floral vibrations

237 at 48kHz sampling rate. This is a well-established and effective method for recording the

238 frequency component of bees' floral vibrations (De Luca *et al.*, 2018). Fresh flowers were used

239 for each bout. Naïve bees, *i.e.* bees with no previous experience of foraging on flowers, were

240 first exposed to *S. citrullifolium* for six consecutive visitation bouts (n = 10 bees), and buzzes

241 in the first and sixth bout were analysed (n = 1,640 buzzes analysed). Then, the same bees were

242 exposed to *S. heterodoxum* for six additional bouts and buzzes in the first (n = 10 bees) and

243 sixth bouts (n=3 bees) analysed (n = 758 buzzes). The lack of a reciprocal treatment (*S.*

244 *heterodoxum,* then *S. citrullifolium*) reflects the reluctance of naïve *B. terrestris* to visit the

245 small-flowered taxon (naïve bees readily visit *S. citrullifolium*). To obtain the fundamental

246 frequency of the floral vibrations produced by bumblebees on *Solanum* flowers*,* we used

247 *Audacity* to obtain the frequency spectrum (FFT) of each floral buzz using a Hamming window

248 (size = 512) and visually identified the fundamental frequency (Morgan *et al.*, 2016).

249

250   STATISTICAL ANALYSES

251 We estimated the correlation among 13 floral traits and natural frequency using Pearson's

252 correlations. We calculated separate correlation matrices for each stamen type (feeding and

253 pollinating) and visualised the results using the package *corrplot* (Friendly, 2002). To analyse

254 variation in natural frequency and stamen characteristics, we fitted a series of linear mixed-

255 effects model with natural frequency as the response variable, stamen length, stamen type and

256 relative flower size (large or small) as fixed effects, and plant taxon ("species") as a random

257 effect using *lme4* (Bates *et al.*, 2015). Stamen length was chosen for analysis because it was

258 strongly and positively correlated with all other floral traits (see Results). Model selection

259 was done by starting with a model that contained all terms plus the interaction 'stamen type *

260 relative flower size' and sequentially removing non-significant terms as assessed by a

261 likelihood ratio test (LRT). The final selected model included both anther length and stamen

262 type. Statistical significance of fixed effects in the final model was assessed with F-statistics

263 with Satterthwaite correction for degrees of freedom, implemented in *lmerTest* (Kuznetsova,

264 Brockhoff, & Christensen, 2017).

265   To analyse the differences within bees' fundamental frequencies, we also fitted a

266 linear mixed-effects model with plant species and bout number as fixed effects, and

267 individual bee identity as a random effect. All analyses were done in *R* version 4.0.2 (R

268 Development Core Team, 2020).

269

270   RESULTS

271   NATURAL FREQUENCIES OF STAMENS

272 Examples of the frequency spectra for feeding and pollinating stamens of two species are

273 shown in Figure 2. Morphological and mass measurements of the 13 floral traits studied here

274 are summarised in Table S2. All measured traits of flower morphology and mass are

275 positively correlated with one another across all flower taxa (Figure 3). The natural frequency

276 of both feeding and pollinating stamens was negatively correlated with all measured floral

277 traits ($\rho <$ -0.11 for all traits; Figure 3), except in the case of pollinating stamens, which

278 showed a very weak positive correlation between frequency and filament major diameter, (ρ

279 = 0.05; Figure 3). Stamen length was strongly correlated with all other floral traits in both

280 feeding and pollinating stamens (Pearson's ρ = 0.37 to 0.95; Figure 3; the numerical values

281 for each correlation are shown in Figure S1). Given the strong correlations among floral

282 traits, we used stamen length in the statistical analyses (linear mixed-effects models) between

283 floral characteristics and natural frequencies.

284   Overall, the average natural frequency of individual stamens across six *Solanum* taxa

285   varied from 44.57 ± 1.36 Hz (mean ± SE) for pollinating stamens of *S. citrullifolium* to

286   294.30 ± 47.37 Hz for the feeding stamens of *S. grayi grayi* (Table 1, Figure 4).

287   Independently of anther type, stamens of large flowered taxa (*S. rostratum, S. grayi*

288   *grandiflorum* and *S. citrullifolium*) had on average lower natural frequencies than their

289   closely related paired taxon with smaller flowers (*S. fructu-tecto, S. heterodoxum,* and *S.*

290   *grayi grayi*) (101.48 ± 20.46 Hz vs. 162.33 ± 25.71 Hz, for large and small-flowered taxa,

291   respectively), although this difference was not statistically significant as assessed by a LRT

292   of nested models with and without flower type (large vs. small; *p*-value = 0.916). The results

293   of the analysis of mixed effects models including taxon, stamen type and stamen length,

294   indicated that stamen type had a significant effect on the natural frequency of stamens.

295   Pollinating stamens had on average lower frequencies than feeding stamens (Table 2; Figure

296   4).

297   Differences between pollinating and feeding stamens were more marked in large

298   flowered taxa and smaller or absent in small flowered taxa (Table 1, Figures 2 and 4), but we

299   did not detect a statistically significant interaction between flower type (large vs. small) and

300   stamen type (feeding vs. pollinating) when comparing nested models using a LRT (*p*-value =

301   0.693). For each pair of closely related taxa, pollinating stamens from the large-flowered

302   taxon had lower natural frequencies than pollinating stamens from its small-flowered relative

303   (Figure 4). Finally, after statistically accounting for species identity and stamen type, we

304   observed a marginally significant negative effect of stamen length on natural frequencies (*p*-

305   value = 0.055; Table 2). In other words, longer stamens tended to have lower natural

306   frequencies than shorter stamens (Table 2).

307

310   We analysed 2,398 floral vibrations of 10 bees visiting flowers of *Solanum citrullifolium* and

311   *S. heterodoxum* (1,640 and 758 floral vibrations in each plant, respectively). All 10 bees

312   visited both plant species at least once yielding 47-279 buzzes per bee per plant species (164

313   $\pm$ 20.99 and 75.8 $\pm$ 14.4 buzzes per bee, for *S. citrullifolium* and *S. heterodoxum*). Two

314   sample floral vibrations of the same individual bee while visiting a flower of each species are

315   shown in Figure 5. We found a statistically significant effect of bout number on the

316   fundamental frequency of floral buzzes (Table 3), but the effect is negligible in *S.*

317   *citrullifolium* (which was visited first; see Methods). In this case, the frequency of floral

318   vibrations in the first bout was 345.25 $\pm$ 0.87 Hz (n=10 bees, 636 buzzes) and 344.04 $\pm$ 0.57

319   Hz in the sixth bout (n=10 bees, 1004 buzzes). The effect of bout was more marked when

320   comparing the first and sixth visit of *B. terrestris* to *S. heterodoxum*. Here, floral buzzes in

321   the first visitation bout had a fundamental frequency of 349.68 $\pm$ 0.70 Hz (n=10 bees, 586

322   buzzes) and 329.47$\pm$0.95 Hz (n=3 bees, 163 buzzes) in their sixth bout. We also found that,

323   overall, a statistically significant, but biologically minor (~5 Hz; Table 3), difference in

324   fundamental frequency among the same 10 bees visiting two morphologically distinct flower

325   types, with bees producing on average lower frequency vibrations in flowers of *S.*

326   *heterodoxum* than in flowers of *S. citrullifolium* (Table 3).

327

328   DISCUSSION

329   In contrast to wind-pollinated plants (Timerman & Barrett, 2018), very little is known about

330   the natural frequency of buzz pollinated flowers, with the notable exception of a study

331   reporting the natural frequency of *Solanum laciniatum* (King & Buchmann, 1996). We have

332   shown here that even closely related taxa in the tomato genus, *Solanum*, have stamens with

333   different natural frequencies. By studying a closely related group of plants in which a single

334   flower bears two morphologically distinct anthers (heteranthery), we were able to show that

335   the difference in this biomechanical property is also captured within the same flower and that

336   it might be associated with the replicate evolutionary shifts in flower morphology observed

337   within *Solanum* section *Androceras* (Vallejo-Marín *et al.* 2014). Variation in natural

338   frequencies of stamens might play a role in regulating patterns of pollen release during buzz

339   pollination, although further experimental work is needed in this area. Consistent with

340   previous work (Switzer *et al.*, 2019), we found little evidence of rapid adjustments in the

341   frequency of floral vibrations produced by captive bumblebees to match the natural frequency

342   of the flowers they visit. However, because the natural frequencies of some types of stamens

343   (feeding stamens of five out of six studied species), overlaps the range of fundamental

344   frequencies produced by other bee species, we suggest that stamen resonance might play a

345   role in facilitating pollen release in some buzz pollination systems.

346

347   HETERANTHERY AND VARIATION IN NATURAL FREQUENCY

348   In heterantherous *Solanum*, anther dimorphism is associated with functional speciation of

349   stamens into pollinator attraction and reward (feeding stamens) and fertilisation (pollinating

350   stamens) (Vallejo-Marín *et al.*, 2009), and we show here that these functional differences are

351   paralleled by distinct natural frequencies between stamen types. The average natural

352   frequency of the feeding anthers of all taxa, except *S. citrullifolium*, are between 100-320Hz,

353   compared with the range of fundamental frequencies of 100-400Hz observed across bee

354   species (De Luca & Vallejo-Marín, 2013; Corbet & Huang, 2014; De Luca *et al.*, 2019; Rosi-

355   Denadai *et al.*, 2020). In contrast, the mean natural frequency of the pollinating anthers of the

356   large-flowered taxa (*S. rostratum, S. grayi grandiflorum* and *S. citrullifolium*) falls below the

357   100-400Hz bee range. The functional consequence of this dissonance in fundamental

358   frequencies among stamen types is unknown. It is possible that some bees may be able to

359   induce stamen resonance by matching their floral vibrations to the natural frequency of the

360   feeding stamens, but the same frequency will not induce resonance in pollinating stamens,

361   potentially controlling pollen dispensing (Dellinger *et al.*, 2019a; Kemp & Vallejo-Marín,

362   2020).

363   For the small flowered taxa (*S. fructu-tecto*, *S. grayi grayi* and *S. heterodoxum*), the

364   mean difference in natural frequency among stamen types is smaller and their ranges largely

365   overlap. This suggests that the evolutionary transition from large to small flowers in this

366   group (Vallejo-Marín *et al.*, 2014) is associated with convergence of vibrational properties

367   between stamen types. Further studies with increased replication in heterantherous taxa with

368   shifts in the expression of anther dimorphism, or during evolutionary transitions between

369   buzz pollination and other modes of pollination (Brito *et al.*, 2016; Dellinger *et al.*, 2019b),

370   might be able to test the hypothesis that evolutionary shifts in stamen function are

371   accompanied by changes in vibrational properties.

372   The effect of stamen type on natural frequency occurs in addition to differences in

373   length associated with the two anther types (which were accounted for in our statistical

374   models) suggesting that other stamen characteristics influence the vibrational properties of

375   different types of anthers. Finer characterisation of stamen morphological properties (e.g.,

376   through analysis of X-ray, μCT scanning as in Dellinger *et al.*, 2019c) as well as their

377   material properties (Mortimer, 2017; Saltin *et al.*, 2019) might help in elucidating the

378   mechanism by which the vibrational properties of these anther types are determined. Because

379   stamens are relatively complex structures and not simple cantilever beams, modelling

380   approaches such as Finite Element Modelling (FEM) (Saltin *et al.*, 2019), could seek to

381   integrate these morphological and material properties to generate predictions of the

382   relationship between floral traits and vibrational properties. Moreover, variation within

383   species (e.g., between varieties of *S. grayi*) also opens opportunities to increase the

384   segregating variation within experimental populations through artificial crosses (Conner,

385   2003), and disentangle the contribution of correlated floral traits to variation in natural

386   frequencies.

387

388   NATURAL FREQUENCY OF INDIVIDUAL STAMENS AND POLLEN RELEASE

389   The overlap between the range of frequencies produced by some bees and those of the

390   studied *Solanum* flowers suggests that in certain taxa, resonance might come into play during

391   buzz-pollination, potentially increasing the magnitude of the vibrations applied by bees and

392   hence, increasing pollen release. A caveat with using our results to interpret the effect of

393   resonance of bee-flower interactions during buzz-pollination is that the natural frequency of

394   the stamen is likely to change as the bee firmly holds the anther with its mandible and

395   presses its body against the stamens as it occurs during buzz pollination (Buchmann, 1983).

396   Future studies will benefit from exploring the resonance of coupled bee-flowers, although the

397   technical challenges to acquire these data are significant. At the very least, the natural

398   frequencies of free stamens we calculated provide an insight into the potential for resonance

399   to increase pollen release in some types of flowers, including heterantherous flowers such as

400   the ones studied here. In many heterantherous flowers, pollinators usually manipulate a

401   subset of the anthers in the flower (feeding anthers) during visitation (Luo, Zhang, & Renner,

402   2008; Vallejo-Marín *et al.*, 2009). In some cases, a set of anthers (usually the pollinating

403   anthers) remains free during floral vibrations (Vallejo-Marín *et al.*, 2009). Furthermore,

404   relatively small buzz-pollinators interact with only some of the stamens even in non-

405   heterantherous flowers (Solís-Montero *et al.* 2015). If the floral visitors vibrate the flower at

406   the natural frequency of the free stamens measured here, then, in principle, resonance at these

407   frequencies could increase pollen delivery during visitation.

408

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

410 If stamen resonance at the natural frequencies is within the reach of, at least some bee

411 species, the question arises as to whether bees exploit this resonance effect during buzz

412 pollination. Empirical work with bumblebees suggests that this is not the case. Our

413 bumblebee experiment suggests that *B. terrestris* does not rapidly match their floral buzzes to

414 the natural frequency of the flowers they visit. Our results are consistent with previous work

415 on bumblebees that also showed a lack of frequency adjustment to match specific vibrations

416 in bees visiting different types of mechanical flowers that released pollen when buzzed at

417 specific frequencies (Switzer *et al.*, 2019).

418   The lack of dynamic adjustment between bee floral buzzes and the natural frequency

419 of stamens over consecutive visits of an individual bee to the same flower could be explained

420 if an individual bee is unable to change the frequency of the vibrations produced during floral

421 visitation. Previous work has shown that the frequency of floral buzzes decreases with

422 experience at manipulating buzz-pollinated flowers, while simultaneously resulting in more

423 pollen being collected per visitation bout (Whitehorn, Wallace, & Vallejo-Marin, 2017). In

424 this case, the change in the fundamental frequency of floral buzzes is relatively small (~20Hz

425 over 10 visitation bouts in Whitehorn *et al.*, (2017), and ~1-20 Hz over six visitation bouts in

426 the present study). Thus, it is possible that there are narrow limits to the adjustment in

427 frequency that a bee can achieve during buzz pollination. However, empirical work has

428 shown that bumblebees can significantly change their buzz frequencies in other types of non-

429 flight vibrations. For example, the defence buzzes produced by *B. terrestris* have a

430 significantly lower frequency (236.32±4.29 Hz) than those produced on flowers (313.09±2.63

431 Hz) (Pritchard & Vallejo-Marín, 2020). This suggests that changes in frequency of non-flight

432 vibrations of larger magnitude (~80Hz) compared to the ones we observed (~20Hz) are at

433   least possible in the same individual bee. The behavioural mechanism that would allow a

434   bumblebee to associate producing a particular buzz frequency with increased pollen release is

435   unclear. Perhaps inexperienced bees initially produce buzzes of variable frequencies and

436   overtime learn to associate particular frequencies with increased pollen release (due to

437   resonance), but the elegant experiment of Switzer *et al.* (2019) with mechanical flowers

438   provides no evidence of this type of instrumental learning.

439   Alternatively, producing vibrations at the resonant frequency of stamens might have a

440   relatively small effect on pollen release compared to the effect of other components of the

441   bee's buzz such as amplitude, and the duration and number of buzzes (King & Buchmann,

442   1996; De Luca *et al.*, 2013). Experiments applying bee-like vibrations of different

443   characteristics show that pollen release is more strongly determined by vibration amplitude

444   (peak velocity) than by frequency in *S. rostratum* (De Luca *et al.*, 2013). The lower-

445   frequency vibrations produced by *B. terrestris* during defence are also lower in amplitude

446   compared to the higher-frequency, higher acceleration amplitude buzzes producing during

447   floral visitation. Vibrations with both low frequency and high acceleration may not be

448   possible to reach by bees if their maximum displacement is reached, e.g., due to the limits

449   imposed by thoracic size (Corbet & Huang, 2014). If the gain in increased pollen release that

450   would be achieved through stamen resonance is offset by a decrease in pollen release due to

451   producing vibrations of lower amplitude, then bees might not benefit from matching the

452   relatively lower natural frequency of flowers. Instead, the optimal frequency of a bee's floral

453   buzz to maximise pollen release may be best explained by the resonance properties of the

454   bee's body. Buzz-pollinating bees may benefit from vibrating at the resonance frequency of

455   their own bodies (King, 1993; King & Buchmann, 2003), which would produce the highest

456   amplitude vibration for a given input of energy.

457

CONCLUSIONS

Stamen natural frequency may not be the most important determinant of the type of vibrations produced by large bees that can reach amplitudes high enough to elicit pollen release. However, exploiting the vibrational properties of stamens may be advantageous for other bees unable to reach the required acceleration amplitudes to elicit pollen release due to small size, mass, or other biomechanical constraints (King & Buchmann, 2003). For these smaller bees, the increase in vibration amplitude potentially achieved through resonance of stamens might allow them to utilise flowers that would otherwise be beyond their vibrational reach. Inducing resonance of poricidal stamens may also be useful as a mechanism to increase pollen release in species of agricultural importance such as tomato (*Solanum lycopersicum* L.), eggplant (*S. melongena* L.) and kiwifruit (*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson), where mechanical shakers are sometimes used to pollinate these crops. Further work should compare the stamen natural frequency of other buzz-pollinated flowers with buzzing frequencies of a broader community of visiting bees to establish whether any bee exploits floral resonance for pollen release. By building on classical work on the biomechanics of buzz-pollination (e.g., King, 1993; King & Buchmann, 1996) our work suggests new and exciting lines of inquiry integrating biomechanics and ecological interactions at the organismal level (Bauer, Poppinga, & Müller, 2020).

DATA ACCESSIBILITY

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

AUTHOR CONTRIBUTIONS

CEPN carried out plant experiments, analysed the data, participated in study design and drafted the manuscript. LN carried out bee experiments, analysed the data, participated in study design and commented on the manuscript. FZM participated in experimental design

482 and commented on the manuscript. MVM participated in conceiving the study and data

483 analysis and helped drafting the manuscript. All authors gave final approval for publication

484 and agree to be held accountable for the work performed therein.

492

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631   FIGURE LEGENDS

632   **Figure 1. (A)** Illustration showing *Bombus terrestris* vibrating the stamens of buzz-pollinated

633   *Solanum rostratum*. **(B)** Diagram of the experimental setup showing the stamen attached to a

634   platform on the magnetic shaker, the direction of the base oscillations, and the position of the

635   laser beam of the Doppler vibrometer.

636

637   **Figure 2.** Frequency spectrum obtained in response to the application of broadband

638   frequency vibrations (white noise; 20-20,000 Hz) applied to individual stamens of two buzz-

639   pollinated, heterantherous species of *Solanum* section *Androceras*. The grey lines correspond

640   to feeding stamens and the black lines to pollinating stamens. The feeding and pollinating

641   anthers of *S. rostratum* (A) are morphologically more distinct than those of *S. fructu-tecto*

642   (B), which is very weakly heterantherous. The first natural frequency corresponds to the

643   lowest frequency peak observed in for each stamen and is indicated for each stamen type with

644   an asterisk.

645

646   **Figure 3.** Visual representation of the Pearson product-moment correlation matrix among 13

647   floral and stamen traits and the natural frequency of stamens across six taxa of

648   heterantherous, buzz-pollinated species of *Solanum section* Androceras. Correlations were

649   calculated separately for pollinating stamens (upper triangle of the matrix) and feeding

650   stamens (lower triangle). Negative correlations are shown in red and positive correlations in

651   blue, with darker colours indicating higher absolute values. The order of the variables shown

652   in the figure were chosen using hierarchical clustering.

653

654   **Figure 4.** Natural frequencies (mean ± SE) of feeding and pollinating stamens of three pairs

655   of heterantherous taxa of *Solanum* section *Androceras*. These six taxa represent three

656   independent transitions in flower size, with one large-flowered, highly heirantherous, and

657   one small-flowered, weakly heirantherous taxon. Each pair of taxa is associated with a

658   different corolla colour. The average fundamental frequency of floral vibrations produced by

659   *Bombus terrestris* on flowers of *S. citrullifolium* and *S. heterodoxum* obtained in this study is

660   shown with a dashed line. The dotted lines show the range of fundamental frequencies of

661   floral vibrations commonly observed across multiple species of buzz-pollinating bees (100-

662   400Hz; De Luca & Vallejo-Marín 2013; De Luca *et al.*, 2019). Flower illustrations depict

663   variation in morphology and preserve size proportions across taxa.

664

665   **Figure 5.** Time and frequency characteristics of floral vibrations produced by the same

666   individual of *Bombus terrestris audax* on buzz-pollinated flowers of *Solanum*

667   *citrullifolium* (A, B) or *S. heterodoxum* (C, D) registered with an audio recorder placed at

668   10cm from the flower. Time domain: panels A and C show multiple buzzes (floral vibrations)

669   produced over two seconds of a floral visit. Frequency domain: panels B and D, show the

670   power spectral density (PSD) of the floral vibration highlighted in purple in the

671   corresponding oscillograms in A and C. The highest peak in the PSD corresponds to the

672   fundamental frequency (333 Hz for *S. citrullifolium* and 332 Hz for *S. heterodoxum*). The

673   first five harmonics of the fundamental frequency are shown with vertical dashed lines (B,

674   D). For plotting, we applied a bandpass filter (50-5000 Hz), and estimated the PSD using a

675   Hamming window (length = 2,048 samples).

676

678

679   **Table 1.** First natural frequency (in Hz) of feeding and pollinating stamens of three pairs of heterantherous *Solanum* section *Androceras*. Each

680   pair of taxa consists of a large-flowered, strongly heterantherous taxon (Large), and a sister-species or closely related taxon with small flowers

681   and weak heteranthery (Small). One anther of each type was analysed per flower.

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

682

683 **Table 2.** Statistical analysis of the effect of stamen type (feeding vs. pollinating) and stamen

684 length (mm) on the natural frequency (Hz) of stamens from three pairs of herantherous taxa

685 in *Solanum* section *Androceras*. Model estimates were obtained from a linear mixed-effects

686 model with taxon as a random effect and stamen type and stamen length as fixed effects.

687 Statistical significance (*p*-values) of the fixed effects were obtained using type III Sums of

688 Squares. SE = Standard error of the estimate.

Model component	Estimate	SE	*p*-value
Intercept	214.559	34.254	
Stamen type (pollinating stamen)	-37.013	13.712	0.008
Stamen length	-7.598	3.755	0.055

689

690 **Table 3.** Effect of plant species and bout number on the fundamental frequency (Hz) of floral

691 vibrations produced by *Bombus terrestris* visiting flowers of two *Solanum* taxa. Model

692 estimates and *p*-values obtained using type III Sums of Squares of the fixed effects of a linear

693 mixed-effects model. SE=Standard error.

Model component	Estimate	SE	*p*-value
Intercept	348.734	3.397	
Plant species (*S. heterodoxum*)	-4.502	0.966	0.008
Bout number	-0.733	0.150	0.002

694
695

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

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

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