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**Buzz-pollination leads to size-dependent associations between
bumblebees and *Pedicularis* flowers**

Authors:

Yuanqing Xu^{1,2}, Bentao Wu³, Mario Vallejo-Marín⁴, Peter Bernhardt⁵, Mark Jankauski⁶,
Dezhu Li^{1,7}, Stephen Buchmann⁸, Jianing Wu^{3*}, Hong Wang^{1,7*}

Affiliations:

¹ Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences; Kunming, 650201, China.

² University of Chinese Academy of Science; Beijing, 100049, China.

³ School of Advanced Manufacturing, Sun Yat-Sen University; Shenzhen, 518107, China.

⁴ Department of Ecology and Genetics, Uppsala University; Uppsala, 75236, Sweden.

⁵ Bayer Herbarium, the Missouri Botanical Garden; Saint Louis, 63110, USA.

⁶ Department of Mechanical and Industrial Engineering, Montana State University; Bozeman, 59717, USA.

⁷ Germplasm Bank of Wild Species, Kunming Institute of Botany, the Chinese Academy of Sciences; Kunming, 650201, China.

⁸ Department of Ecology and Evolutionary Biology, and Department of Entomology, University of Arizona; Tucson, 85721, USA

*Corresponding authors: Hong Wang, Jianing Wu.

Authors' emails:

xuyuanqing@mail.kib.ac.cn, wubt3@mail2.sysu.edu.cn, mario.vallejo-marin@ebc.uu.se,
bernhap3@gmail.com, mark.jankauski@montana.edu, dzl@mail.kib.ac.cn,
buchmann.stephen@gmail.com, wujn27@mail.sysu.edu.cn, wanghong@mail.kib.ac.cn.

Corresponding authors' emails and telephone:

Hong Wang: wanghong@mail.kib.ac.cn, +86 13078743754;

Jianing Wu: wujn27@mail.sysu.edu.cn, +86 13060867814.

Author contributions:

Conceptualization: YQX, HW, JNW, MVM, DZL

Methodology: YQX, JNW, BTW, MJ, HW

Investigation: YQX, HW

Visualization: YQX, BTW

Writing - original draft: YQX, PB, MVM, HW, BTW

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47 **Abstract**

48 An estimated 20,000 angiosperm species conceal pollen inside tubular poricidal anthers or
49 within modified petals. A bee releases pollen by vibrating its thorax and transferring the force
50 through its mandibles while biting the flower. While the floral morphology of buzz-pollinated
51 plants is diverse, the behaviors, dimensions and guilds of buzzing bees are limited. Floral
52 modifications should reflect the relative sizes of their co-adapted pollinator species but we do
53 not know what drives these size associations. We show that the optimal excitation point in the
54 vibration system of bumblebee-pollinated louseworts (*Pedicularis*) occurs precisely where
55 bumblebees bite these flowers. This leads to trait matching between a bumblebee's individual
56 body length and the beak length of the flowers of each lousewort species. As bumblebees do
57 not visit flowers with beaks (galea) longer than their bodies it guarantees they bite the optimal
58 excitation point for pollen discharge.

59

Main Text:

Buzz-pollination is a specialized but widely distributed pollination syndrome throughout angiosperms (1). Pollen foraging insects (bees and a few flies) apply thoracic vibrations to harvest pollen from flowers concealing pollen in tubular/conical structures (sensu (2)). More than 20,000 angiosperm species, representing about 70 plant families are thought to be buzz-pollinated (1, 3). Those plants rely upon at least 74 bee genera capable of buzzing these modified flowers (4) while they grip floral organs with their mandibles and tarsal claws (5, 6). Pollen grains inside hollow poricidal chambers are shaken out of their anthers via terminal openings to be deposited on bees' bodies, then transferred to hind legs or abdomens and carried back to nests to provision offspring or siblings. The convergent evolution of buzz pollinated flowers occurs in many plant groups(3) and might be "mimicked" by some orchid species (*Calopogon*, *Thelymitra*) that make false anthers and bee visitors vibrate these structures without receiving any reward (7). Many economically important crops including tomato, eggplants, kiwi fruit, and blueberries are buzz-pollinated (8).

The morphology of buzz-pollinated flowers varies in organography, structure, and overall flower size (9). The flowers of congeners may present different numbers of poricidal anthers of divergent lengths and shapes (e.g., *Solanum* (10, 11)). Others show fusion (connation) of anthers forming anther cones (e.g., *Echeandia* (12) and *Solanum*(11)). In other cases, some species in the genus *Pedicularis* retain longitudinally dehiscent stamens within a galea, an elongated and tubular hollow beak composed of connate petals (sensu (13)). It was suggested previously that variation in flower form in buzz-pollinated species has the potential to allow unrelated plant species to exploit the enormous variation in bee size and behaviours among different bee species sharing the same habitat (14, 15). Previous studies showed that flower size predicted body lengths of the most frequent species of bees pollinating flowers with poricidal anthers (16). In addition, *Bombus friseanus* shows intraspecific variation in body sizes leading to assortative foraging on different, buzz-pollinated *Pedicularis* species (17). It remains unclear, though, as to which mechanism drives such size-dependent correlations between bees and their buzz-pollinated host plants.

Buzz-pollination is interpreted as a forced vibration system, where the force input is the bee's thoracic buzz while the vibration of the flower is the system response. Floral vibration is affected by the physical properties of each flower and characteristic of the excitation force (bee vibrations) including buzz frequency, duration and amplitude (5). The bee's mass loading and the excitation point should impact the system's vibration dynamics and transmission as well (18, 19). Nevertheless, the points at which the bee's vibrations are transmitted to the flower (the excitation point) has not been studied in detail. Although a bee's vibrations are generated by its thoracic muscles, these forces should be transmitted to the flower at the point where the bee bites the flower due to the immediate connection between its head and thorax (6). Of greater importance, as grains are released from the terminal openings of pollen-concealing structures (e.g., the terminus of the *Pedicularis* petal beak) during vibration, a bee presses its body to those openings to collect ejected pollen directly onto the ventral side of its abdomen (20). Consequently, the location of the biting point and the dimensions of the pollen release structure may confine a bee's choice of flowers depending on its own body size.

We hypothesize that the excitation point (that is, where the bee bites and applies forces to the flower) plays a critical role in the vibration amplitude of the flower. It could be the mechanism driving a bee's floral preferences. We used *Pedicularis* L. (Orobanchaceae) as our model because it is one of the largest angiosperm genera with over 600 species in the Northern Hemisphere (21). The genera *Pedicularis* (louseworts) and *Bombus* (bumblebees) have their centers of diversity in the Himalaya-Heng Duan Mountains region (22). Almost all lousewort species are bumblebee pollinated, and sympatric species in this floristic region often form co-blooming swarms sharing the same *Bombus* species as their primary pollinators (23-26). Bumblebees usually vibrate only beaked flowers of *Pedicularis* species for pollen, and forage for nectar or pollen on sympatric but beak-less congeners (27). The floral diversity of beaked species in this genus is higher than in beak-less species (21). The tube-like beak fulfils a similar function to hollow, elongated and poricidal anthers in unrelated buzz-pollinated species, as pollen grains are released at the terminus of the beak following vibration (Fig. 1A). Beak morphology is more variable than poricidal anthers in terms of dimension and/or shape (Fig. 1C), making it an ideal model lineage to study interactions between bumblebees and buzz-pollinated flowers.

To test our hypothesis, we first recorded bumblebee behaviors and biting points, on eight buzz-pollinated and Chinese *Pedicularis* species (Fig. 1D). We then developed computational finite element models to assess the effects of different locations of excitation points and different bee sizes affecting floral vibration under the same vibration modes. As the amplitude of bee-mediated vibrations are the main determinants of the amount of pollen released when flowers are buzzed (5, 28) it became the main dependent variable we used during simulations. We then analyzed the relationship between bee body size and floral traits *in situ*. By combining behavior observations, computational modelling, lab vibration experiments and an ecological statistical survey, we now clarify the mechanism(s) driving any size-dependent correlations between bee and flower morphology in buzz-pollinated congeners.

How bumblebee harvest pollen from beaked *Pedicularis* by vibration?

We assessed the behaviors of worker caste bumblebees recorded from 113 video clips. Although these *Pedicularis* species show different modes of floral presentation, our videos show that buzzing behaviors (floral sonication) of bumblebees and their biting points were similar regardless of plant species or field site. (Fig. 2A-G). Bumblebees generally land on the left side of the flower beak. All biting sites on eight *Pedicularis* species occur where the beak connects to the body of the galea (Fig. 2H). Female bumblebees use their front and middle legs to clasp the beak and press its abdomen to the beak's terminal opening. The same bee's thorax almost never touches the beak. When bees begin to vibrate, the pollen released from the beak is deposited on its abdomen. As this occurs, the bee uses its hind legs to collect pollen grains ejected at the beak's terminus onto its abdomen (Video1). A typical bite and sonication last 2-4 seconds leaving an indentation (bite mark) on the galea lobe (Fig. S1). The self-consistency of all buzzing and collection behaviors suggests that a bees' choice of biting sites does not reflect random selection.

Why do bees bite at this location?

The biting point of a bumblebee marks where vibration transfers to the flower. Therefore, we applied computational modelling (Finite Element Analysis, FEA) to study how biting locations affect the bee-flower vibration system. We crafted a shell-structured *Pedicularis* flower model (Fig. 3D), incorporating a straight beak, an oblate tube, and an ellipsoidal galea

(details in SI). To make the model match a nature flower, the geometric characters were determined by anatomical studies with Mirco-XCT and measurements based upon fresh flowers (Fig. 3A-C). Material properties (density and Young's modulus) were based on experimental findings. Prior to simulations, the flower model's natural frequencies and mode shapes were scrutinized to prevent resonance induced by the 300 Hz bumblebee floral buzzing frequency (Fig. S2). Validation occurred through lab vibration experiments on *P. integrifolia* flowers, mirroring our geometric model (Fig. S3). The FEA model's excitation direction aligned with bumblebee visitors' vibration direction.

We conducted two distinct FEA analyses, investigating the impact of various factors on floral vibration. The initial examination focused on the influence of different excitation points on anther vibration, while the subsequent analysis explored the effects of bumblebee size on floral vibration. In the first phase, our findings revealed that applying excitation at point 7, situated on the base of the galea's beak near the anther cluster, led to maximum anther amplitude (refer to Fig. 4B). Consequently, we identified point 7 as the optimal excitation point for our flower model. This conclusion was corroborated by a laboratory vibration experiment on fresh flowers, demonstrating heightened beak movement amplitude when excited at this location. Remarkably, this optimal excitation point on the geometric flower model aligned with biting indentation marks observed on louseworts at field sites left by bumblebees. In the second FEA treatment, we explored the impact of different bumblebee sizes on the floral vibration system. Loading a bee's mass onto the system resulted in alterations to natural frequencies and vibration modes. Notably, when a bee matched the flower's beak length and bit the optimal location on the galea, it induced the most significant beak and anther vibration displacement, even with identical vibration frequency and amplitude (refer to Fig. 4CDE). Given the positive correlation between pollen release and amplitude (29, 30), our results suggest that a bee biting at the optimal excitation point should receive more pollen compared to bees with varying body lengths. Therefore, according to FEA outcomes, a bee's selection of the optimal biting point can be interpreted as the most efficient and economical behavior to maximize pollen release and subsequent harvest.

What are the consequences of a flower's optimal biting location?

To detect whether the optimal biting location effects interactions between bumblebees and louseworts *in situ* we collected and identified 179 individuals representing 9 bumblebee species found foraging on all 8 *Pedicularis* species (see the frequency of interactions in Fig.5). Three bee body traits and 6 floral traits were measured (Fig. S4&5). Their mean values are listed in Table S1. At the species-level, fourth-corner analysis of trait matching indicated a marginally significant matching ($p < 0.05$) occurring between the inner- and outer- linear length of the galea beak with bee body length and the ITD of each bumblebee species. At the individual level, firstly we compared the frequency distribution of the ratios of each flower traits to bee's body length. Overall, only the distribution of trait-pairs relating to beak present bell-shaped curve, suggesting that rather than flower tube length and lip width, bumblebee interacts with flower species mainly influenced by the traits of flower beak (Fig.6). Despite the four traits of beak are self-correlation, the results show that the distribution of ratio between in-linear beak length to bee body length has highest density and sharper peak. This suggests the distance between biting point to beak opening end effected interactions frequency between bee and flower more strongly. The ratio of this trait pair distributes bias less 1.0, indicating bumblebee prefers to visit the flower with beak length shorter to its own body length. Secondly, we found evidence of a significantly positive relationship between bumblebee body length and the length of the galea beak (GLMM: $F = 6.3501$, $p < 0.01$), suggesting trait-matching between flowers and their bee visitor individuals.

This matching of bee body length to beak linear length at the individual level suggests that the optimal biting location affects interactions between bumblebees and louseworts under field conditions. The beak's linear length is the distance from the base of the galea (optimal excitation location) to the beak's terminus. This corresponds to the distance from the bee's mandibles (biting structures) to its abdomen. In the field, bumblebees attempt to collect pollen released from the terminal opening of any *Pedicularis* beak on their abdomens. Therefore, if pollen settles on a bee's body where it cannot groom itself (so-called "safe sites"), or if grains are discharged into the air missing the bee's body, then pollen collection efficiency must decrease (31). When a bee applies its mandibles to this optimal area, while holding its body simultaneously to gather the pollen discharged from the beak's terminus, its body length becomes a constraint on its ability to receive harvested *Pedicularis* pollen just as its' proboscis

length constrains its consumption of nectar from elongated floral tubes and spurs in other angiosperm lineages (32). Therefore, by combining the results of computational model analyses, such trait matching can also be interpreted as another consequence of foraging economics and bee decisions. Our study provides evidence that the excitation point plays a major role in buzzing behavior, potentially by mediating size-dependent constraints between bees and buzz-pollinated flowers (16, 17), in which bee size is related directly to the deposition of pollen on specific parts of a forager's body.

The contribution of buzz pollination to floral constancy

Lousewort species are generally sympatric, co-blooming and share the same bumblebee species pollinator (27). Our observations and bee collections also showed that co-blooming *Pedicularis* species shared the same bumblebee species (Table S1). However, hybridization is relatively rare in this genus although species swarms occur throughout the Northern Hemisphere (23, 24). Identification of prezygotic isolating mechanisms in *Pedicularis* remain incomplete (25). Previous authorities suggested that pollinator floral constancy is the key pre-pollination barrier in *Pedicularis* (33, 34). How this mode of floral constancy evolved in the predominantly bumblebee pollinated lineages of *Pedicularis* is unclear as bumblebees are generally broad (polylectic) pollen foragers (35). A recent study found a few bumblebee species' flower preferences were sometimes modulated by the relative floral abundance of co-flowering and pollen-rewarding *Pedicularis* (36). However, how individual bumblebees' floral constancy evolved in these louseworts remains unknown.

Our results reveal more about issues concerning likely isolating mechanisms. As the optimal excitation location forces a bumblebee to choose one *Pedicularis* species based on its own matching body size, we argue that buzz pollination may facilitate floral constancy by functioning as an isolating mechanism among co-blooming members congeners. During a foraging bout, individual bumblebee workers, rarely foraged on more than one *Pedicularis* species, lowering the possibility of interspecific crosses by carrying heterospecific pollen loads. Flower constancy is affected by a combination of floral and pollinator traits (37-39). Bumblebee workers produced in the same nest vary greatly in physical parameters (e.g., body length, thorax width, proboscis length) over one season (40). The body lengths of bumblebee

workers from the same colony restricts their choice of *Pedicularis* pollen hosts based on the best excitation point and beak variation among flowers of co-blooming species. In turn, individual bumblebees belonging to different species but with similar body lengths can pollinate the same *Pedicularis* species. This kind of morphological matching should promote pollinator niche partitioning (41), while reducing heterospecific pollen deposition.

Learning how to forage for pollen represents a substantial time investment for individual foragers (42) and buzz pollination represents a complex skill. During field observations, we saw that the pollen foraging of some bumblebees on flowers showed less skill than others. Therefore, with multiple *Pedicularis* species, each forager must learn where to bite to find the best excitation point. Although naïve bumblebee workers of *B. terrestris* and *B. impatiens* know instinctively how to sonicate during their first or second visits, they make numerous attempts at fine-tuning their buzz frequencies and amplitude to achieve optimal pollen collection on *Solanum rostratum* (43). That compels an experienced bee to visit flowers it has learned to manipulate (44). The economic decision on which flowers to forage facilitates patterns of floral constancy (45, 46) and may explain why pollen-foraging bumblebees are more constant to buzz-pollinated flowers compared to sympatric, nectar-secreting flowers (47).

Floral diversification within the same, specialized pollination mechanism

Our findings contribute to understanding a key problem in flower evolution. How does floral diversification evolve in congeneric plant species dependent upon the same pollinators? Our study supports the idea that changes in floral presentation in a buzz-pollinated lineage allow sympatric species to exploit broad variations in bee sizes and behaviors (4). The pattern we observed occurs on bees' size at the individual level rather than at the species level. In view of the role of buzz pollination to floral constancy, diversification of flower morphology among *Pedicularis* species may be a mechanism employed in a much broader mode of ethological isolation at the individual level. Although reproductive isolation between the beakless (nectar secreting) and beaked flowers is predicted to occur by mechanical isolation alone (48), bumblebee visitors still showed a strong floral constancy when these two floral forms are sympatric and co-flowering (33, 34). Therefore, ethological isolation remains the main mode of interspecific isolation among *Pedicularis* species regardless of floral forms. Although

louseworts are pollinated exclusively by the local *Bombus* guild, the galea form of a lousewort flower partitions bumblebee behavior into buzzing if the flower has a beak, or nectar foraging if the flower is beakless. In addition, variation in floral morphology among louseworts with beaks partitions bumblebees foraging according to their body sizes, buzzing skills, and learning abilities.

The role of the excitation point within interactions between bees and plants with true poricidal anthers

Matches between the corolla size of flowers and their pollinator's body sizes occur in some of the more common buzz-pollinated plants with poricidal anthers (16). We wondered if the excitation point also acts as a selective mechanism driving size-dependent correlations between highly modified and poricidal anthers and their bee visitors? In our FEA simulations and lab experiments, we found that vibration applied at the optimal biting site can excite the maximum back and forth amplitude displacements of the whole flower, especially the open ends of its beak. This indicates that the excitation point is important for the vibration response of poricidal flowers, if we regard the beak of a *Pedicularis* flower as comparable to one tubular poricidal anther. Research on poricidal anthers shows that the location and magnitude of bee mass loading onto these organs changes the natural frequencies and vibrational modes (19). Shifts in the location of the biting point causes changes to the center of bee mass on poricidal flower structures, influencing the vibrational response of an anther during bee sonication. Therefore, the excitation point likely plays a role in mediating size-dependent correlations between bees and the beaked galea of certain *Pedicularis* species.

Flowers with poricidal anthers show morphological diversity in staminal architecture (fused or free), filament length, and the presence or absence of filamentous appendages or exaggerated sculpturing of the stamen's epidermis (2). All these modifications likely influence vibration transmission and pollen release under buzzing (11, 49, 50). It is reasonable to presume that flowers with different structures possess relevant excitation points and/or areas associated with maximum vibration transmission and response. Applying computational model analysis on other buzz-pollinated angiosperms will help us understand how excitation points may affect those similar vibration systems.

Benefits and limitations of computer-based models to buzz-pollination vibration analyses

Finite element analysis (FEA) is a reliable and tested computational model. This method has been used to resolve many biomechanical issues, especially those involving functional morphology (51, 52). With this tool, we suggest the crucial role of an optimal excitation point of the vibration system of bee-pollination in *Pedicularis*. This, however, does not fully explain the vibratile pollen-release mechanism. Beaks in *Pedicularis* and poricidal anthers in other, unrelated species vary in length, shape, curvature, ornamentation, degrees of twisting and in other morphological/physical factors. The functions of such modifications remain largely unknown. Applying FEA studies to relevant geometric models is advantageous when investigating how these modifications may influence floral vibration dynamics.

As the objective of any foraging bee applying vibration is to harvest pollen, the pollen release process is important to understand how the pollinator's behavior, is influenced by numerous floral traits, (5, 11, 50, 53). Unfortunately, our FEA results alone cannot be applied to the motion of pollen as the behavior mode of these grains during vibration. For this, we need different methods to study the movement of pollen grains under vibration. Previous models presented the anther as a tall rectangular box with an opening hole (53). Reinterpretation is required as we now understand that the poricidal anther of *Solanum*-type flowers (2) since the beak of *Pedicularis* flowers are more like hollow cylinders. The movement of pollen grains is also more similar to particle motion within a vibrating tube (54, 55). Indeed, numerical simulations based upon the discrete element method (DEM) have since been applied successfully to the study of particles (e.g. powders) moving along a vibrating tube (56). Numerical simulation could also be a powerful computational modeling method for studying pollen ejection during buzz pollination.

By combining multiple disciplines, our study is the first to implicate the role, and importance, of the floral excitation point in buzz pollination. This provides a new insight for our understanding of size-dependent matches between differently sized bees and flowers. Due to the optimal excitation point, modification of floral traits in one lineage of buzz-pollinated plants encourages exploitation of pollinators of different sizes at the individual level but not at the species level. Such trait matching may further contribute to ethological isolation while

lessening competition among co-blooming buzz-pollinated species dependent upon the same pollinator guild of bumblebees. As buzz pollination is a very specialized plant-pollinator mechanism, our study indicates that the role of individual foragers may be crucial contributors to floral diversification. It may provide further insight about how we interpret the evolution and maintenance of relatively narrow and specialized patterns of mutualistic interactions among flowering plants and their bee pollinators.

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Supplementary Materials

Materials and Methods

Supplementary Results

Figs. S1 to S6.

Tables S1 to S2.

References (57–66).

Video 1.



491 **Figure 1. Flower forms of *Pedicularis*.** A. Flower organography with beaked galea showing the
 492 receptive stigma protruding from the galea's beak (drawn by Xu, YQ); B. Flower forms of bilabiate
 493 species (galea lacks a beak); C. Variation in floral forms of beaked species (Flora Yunnanica. tomus-16,
 494 2005); D. flowers of eight species used in this in this study, bar = 10mm. a. *Pedicular oxycarpa*; b. *P.*
 495 *cephalantha*; c. *P. longiflora*; d. *P. milliana*; e. *P. rhinanthoides*; f. *P. axillaris*; g. *P. integrifolia*; h. *P.*
 496 *gruina*.



497 **Figure 2. Foraging positions and biting points of bumblebees during buzzing seven *Pedicularis***
 498 **species. (A to G) *B. festivus* on *P. cephalantha*; *B. nobilis* on *P. integrifolia*; *B. friseanus* on *P. gruina*;**
 499 ***B. friseanus* on *P. oxycarpa*; *B. festivus* on *P. rhinanthoides*; *B. friseanus* on *P. longiflora*; *B. ladakhensis***
 500 **on *P. milliana*; H1&2. Flowers of *P. rhinanthoides* shows galea structure and anther cluster. Arrows**
 501 **indicate the locations of the biting points.**

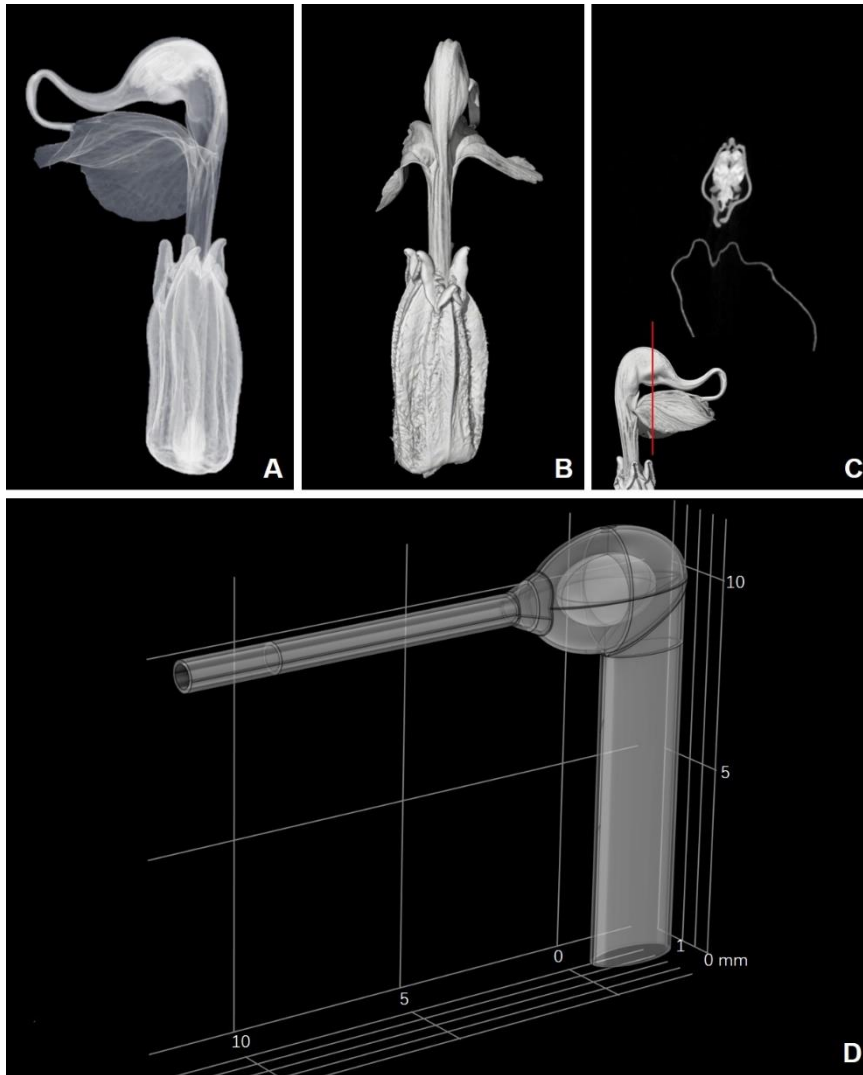


Fig. 3. Morphology of real beaked *Pedicularis* flower and geometric model flower. A, B&C Micro XCT images of *Pedicularis integrifolia* as example showing the translucence view, back side view, and cross section and close-up of anther cluster. D. Geometric model for FEA. Specifically, the shell thickness was 0.1mm, the beak was a 10 mm long and 0.8 mm wide tube, the galea's shell was 4.9x2.1x3.2 mm, the anther ellipsoid was 2.8x1.8x2.8 mm and the flower's oblate tube is oblate in shape 1.2x2.5 mm.

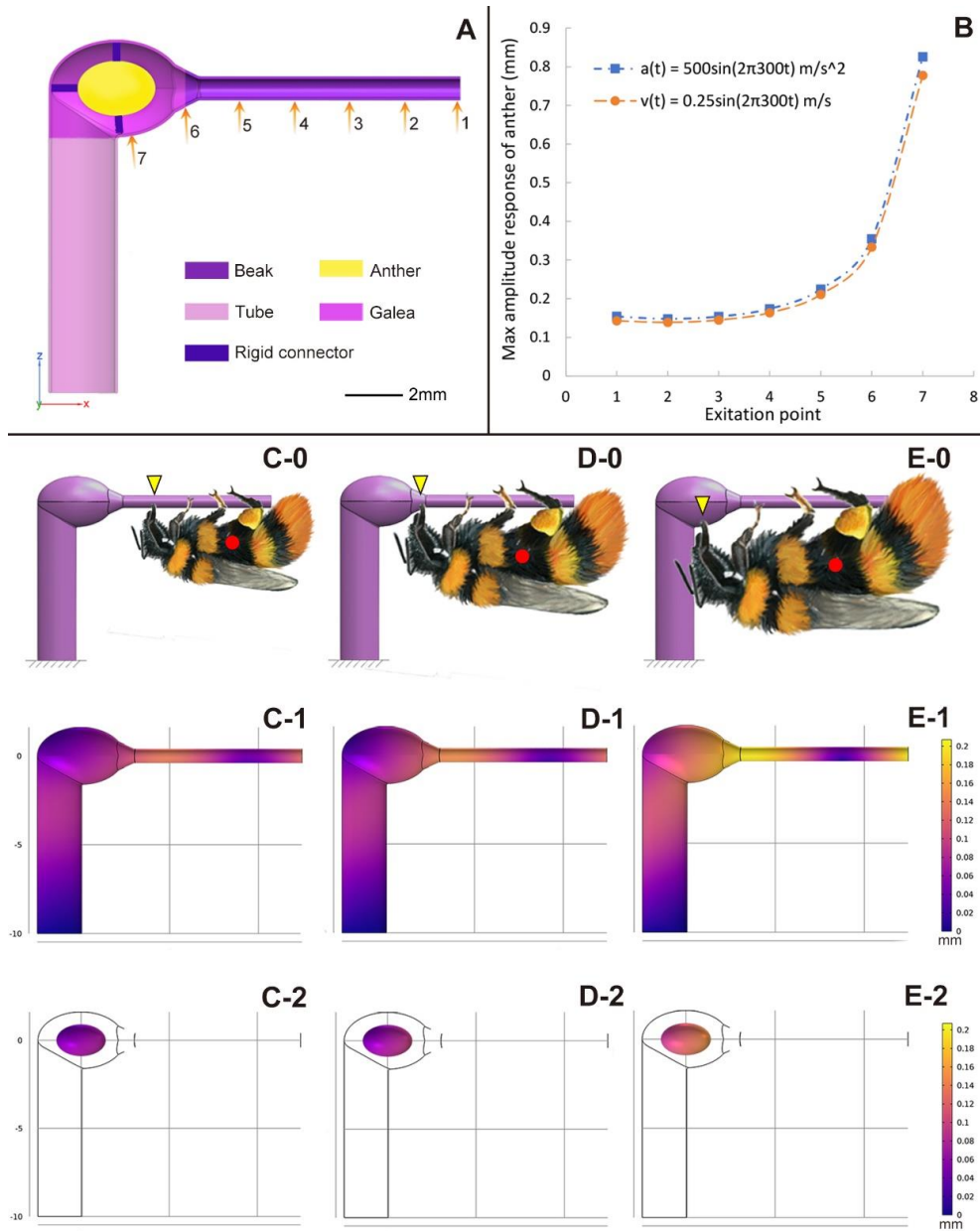


Figure 4. The effects of biting points on the bee-flower vibration system of beaked *Pedicularis*. **A** The computational model of a beaked *Pedicularis* flower. Arrows show the excitation points' location and direction. Bar = 2mm. **B** The maximum amplitude of the anther ellipsoid in the z direction of each point under excitation. **(C to E)**. Vibration response via FEA for *Pedicularis* flowers with bees of different sizes (C. Small, D. Medium, E. Large) under the same forcing vibration function. Δ and $\circ$ show the excitation point and center mass respectively. C-1 to E-1 are the responses of the respective flower with C-2 to E-2 as the responses of the respective anther ellipsoid.

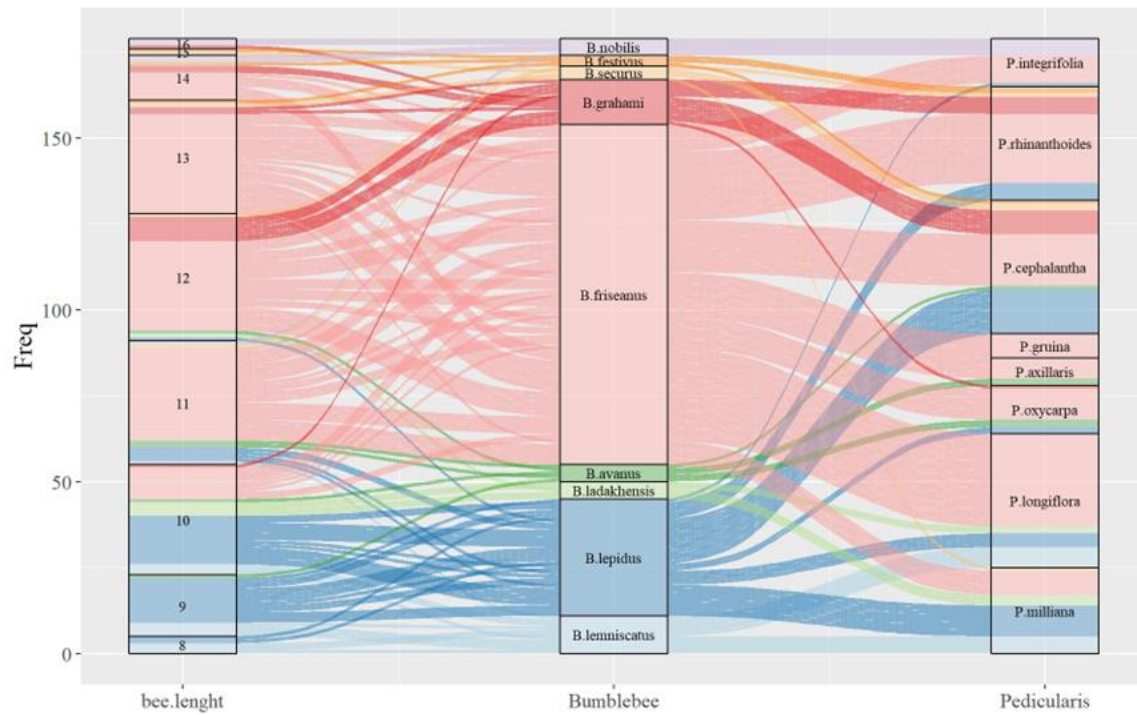


Figure 5. The bee individual length (mm) distributions of each visiting bumblebee species and their interaction frequencies with each *Pedicularis* species. From top to bottom, bumblebee species are ranked by mean body length and *Pedicularis* species are ranked by mean beak in-linear length.

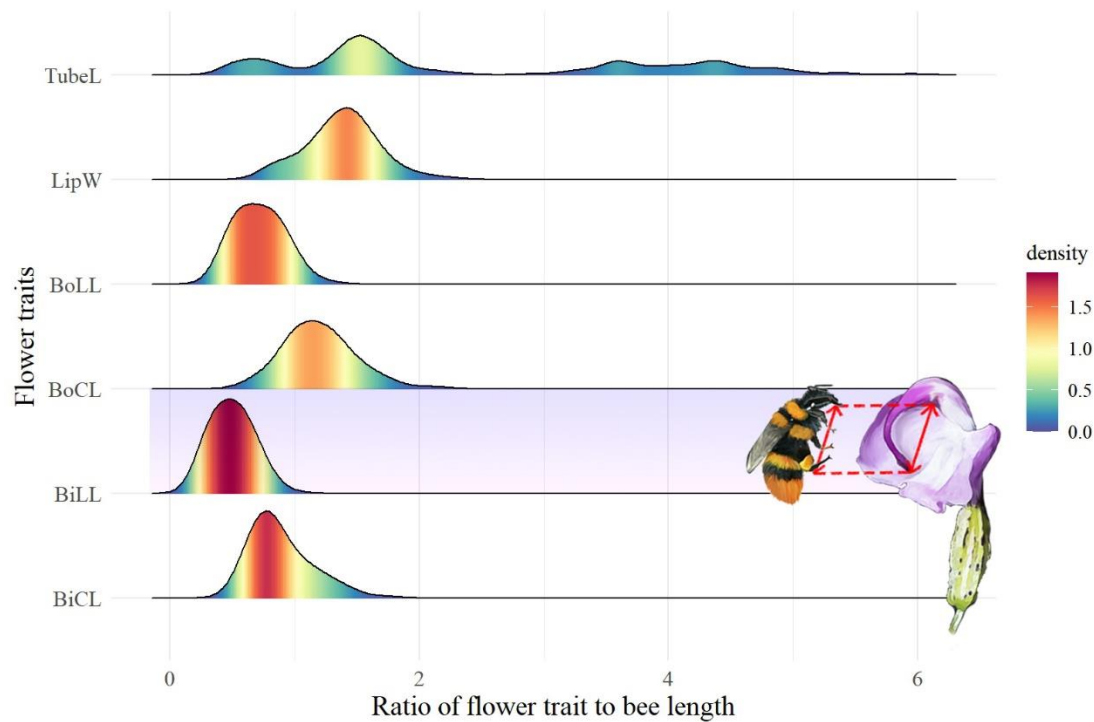


Figure 6. Distribution of trait ratios between flower traits of *Pedicularis* species to body length of visiting bumblebee individuals fitted by kernel density estimation method. TubeL: flower tube length, LipW: low lip width, BoLL: out linear length of flower beak, BoCL: out curve length of flower beak, BiLL: in linear length of flower beak, BiCL: in- curve length of flower beak. Polygon shows the matching trait pair.