Article

GETTING HOOKED? TESTING THE FUNCTION OF ANTHER SPURS IN *VACCINIUM MYRTILLUS*

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Abstract—Flowers display remarkable diversity, much of which is shaped by interactions with pollinators. While various floral traits are considered adaptations to pollination, the function of many morphological features remains untested. In buzz-pollinated species, where bees use vibrations to extract pollen, anther morphology is diverse and can influence pollen removal and transfer. Poricidal anthers, common in these species, sometimes bear spur-like projections. These spurs are hypothesized to aid pollen release, but this has not been experimentally tested. Here, we investigated the function of anther spurs in *Vaccinium myrtillus* (Ericaceae). Using a laboratory setup, we conducted two complementary experiments: one where *Bombus terrestris* bumblebees foraged on flowers, and another applying 1 s artificial vibrations to mimic bee buzzes. We compared pollen release between flowers with intact anther spurs and those where spurs had been removed. Bumblebees produced vibrations during 90% of visits, irrespective of spur presence. Buzzing visits were shorter (21.70 ± 24 s; mean ± SD) and removed more pollen (60 ± 29%) than non-buzzing visits, which were three times longer (63.79 ± 70.45 s) but removed 23 ± 43% of pollen. Artificial 1 s buzzes removed an average of 23% of pollen. Spur removal increased pollen release in both the bee (54% to 60%) and the artificial buzzing experiment (18% to 28%). Our results suggest that anther spurs in *V. myrtillus* potentially act as a pollen-dispensing mechanism, but this effect depends on bee behaviour and visit duration. Further studies could explore other roles the spur has in interactions with non-buzzing visitors, such as regulating pollen release when visitors collect nectar.

Keywords—Anther morphology, *Bombus*, buzz pollination, pollen removal, spur, *Vaccinium myrtillus*.

INTRODUCTION

Flowers are the most diverse plant organs, a diversity partly explained by the interactions they form with their pollinators (Dafni 1984; Campbell et al. 2010; Hempel De Ibarra et al. 2015; Trunschke et al. 2021). Form and function are often related, and examining flower morphology can reveal much about the plant's interactions with other species (Buchmann 1983; Galen 1989; Muchhala 2007; Schiestl & Johnson 2013). Morphological adaptations like colour, size, and scent have been investigated in several systems (Galen 1989; Bradshaw & Schemske 2003; Raguso 2008). However, other floral structures that can influence pollination, such as the anthers, have received far less attention, even though their traits may hold important functional value. Given that anthers are an important site within the flower where the interaction with the pollinator occurs, several aspects of anther morphology may influence the outcome of the interaction with the pollinator.

A clear example of the anther serving as a key trait for pollinator interaction is seen in buzz-pollinated flowers, where the anther's structure regulates pollen release in response to vibrations produced by visiting bees. These flowers often possess tube-like anthers with a narrow opening at their tip (hereafter poricidal anthers) containing the pollen, which is released when a bee vibrates the anther (Michener 1962; Buchmann 1983; Vallejo-Marín 2019). Typical buzz pollinated flowers have large anthers displayed prominently at the centre of the flower, making it easy for bees to land and grip them while performing their vibrations (Faegri 1986; Vallejo-Marín et al. 2022). In other types of buzz-pollinated flowers, such as in bilberry (*Vaccinium myrtillus*), the poricidal anthers are surrounded by the corolla, and the buzz-pollinating bee applies vibrations to the corolla, indirectly vibrating the anthers. In both cases, the structure and positioning of the anthers play a crucial role in mediating the interaction between flower and pollinator, illustrating their importance as a key trait in buzz-pollination systems.

Some buzz-pollinated flowers have appendages connected to the anther (Fig. 1), although their role in pollen release and reproductive success has been rarely tested (Bochorny et al. 2021). Anthers with appendages are commonly found in the families Ericaceae (Judd 1979) and Melastomataceae (Renner 1993; Melo et al. 2022). In Ericaceae, they occur in at least seven different genera (*Vaccinium, Erica, Gaultheria, Calluna, Bejaria, Pieris, Enkianthus*) (Judd 1979; Hermann & Palser 2000), while in Melastomataceae, they are present in at least seven different tribes (Dissochaeteae, Lavoisiereae, Marcetieae, Melastomateae, Merianieae, Rhexieae, and Pyramieae) (Renner 1993; Clausing & Renner 2001; Michelangeli et al. 2013; Da Rocha et al. 2018; Kartonegoro et al. 2018; Bochorny et al. 2019). These appendages can originate from different parts of the stamen and vary widely in shape, size, and colour, with forms ranging from flattened, robust, slender, and fragile (Fig. 1) (Judd 1979; Melo et al. 2022). In both Melastomataceae and Ericaceae, appendages have evolved and been lost independently multiple times (Judd 1979; Melo et al. 2022), suggesting that their presence may confer an adaptive advantage for pollen presentation and pollinator interaction (Judd 1979).

Anther appendages in buzz-pollinated flowers have been hypothesized to serve several functions that mediate plant–pollinator interactions. These functions include (1) attracting pollinators, either visually or by providing a food reward; (2) influencing pollen release by increasing or decreasing pollen output in response to vibrations; (3) triggering pollen release during nectar feeding; and (4) serving as a site for secondary pollen transfer. For example, in many Melastomataceae, appendages are thought to visually attract pollinators and aid in positioning them on the flower to improve pollen placement (Renner 1989; Luo et al. 2008). In *Axinaea*, the appendages provide a direct food reward for pollinating birds

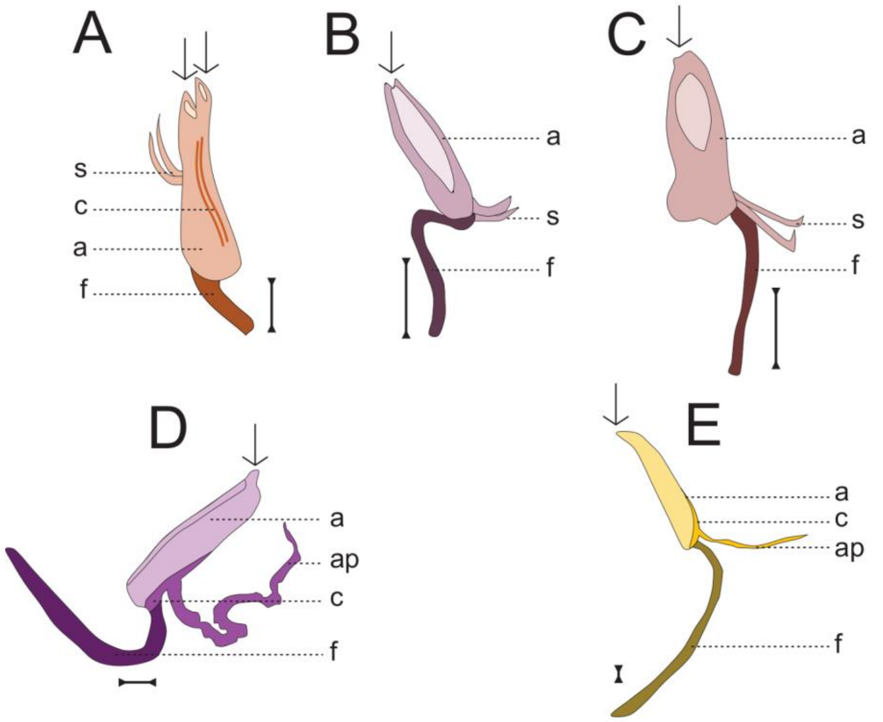

Figure 1. Examples of anthers with appendages in different species of Ericaceae and Melastomataceae. Ericaceae: (A) *Vaccinium myrtillus*, (B) *Calluna vulgaris*, (C) *Erica tetralix*; Melastomataceae: (D) *Huberia bradeana* and (E) *Huberia insignis*. Scale bar: 0.5 mm. Different structures are illustrated with different colour tones and with letters where a = anther cone, c = connective tissue, f = filament, and s = spur, ap = appendage. Notice that all anthers have an appendage: In A-C, the appendage originates from the anther wall and is called a spur, whereas in D and E it originates from connective tissue and is simply called an appendage. The arrows are pointing to the pore(s) of the anther. Illustrations adapted from Moquet, Bruyère, et al., 2017; Bochorny et al., 2021; Passos et al., 2022.

(Dellinger et al. 2014). Regarding pollen release, appendages in *Huberia bradeana* enhance pollen expulsion by increasing vibrational amplitude (Bochorny et al. 2021), whereas in *Huberia insignis,* the appendages do not affect pollen release (Passos et al. 2022). From the flower's perspective, limiting pollen release during a single visit could be a strategic component of its pollen dispensing schedule, promoting pollen distribution across multiple flower visitors (Harder and Thomson 1989, Harder & Wilson 1994). Conversely, a pollen-collecting flower visitor may attempt to maximize its pollen intake per visit, potentially undermining the plant's pollen-dispensing strategy. Therefore, morphological traits that influence pollen release, such as anther appendages, could be subject to selection depending on how they mediate this conflict between plant and pollinator interests. In some Ericaceae species, appendages are hypothesized to play a role in nectar-mediated pollen release, where solid anther spurs may act as obstacles to the pollinator's proboscis during nectar feeding, triggering pollen expulsion (Müller 1883, p.377; Judd 1979; Dorr 1981). Alternatively, they might function as a site for secondary pollen transfer, allowing pollen deposited by a buzzing visitor to be picked up by a subsequent nectar visitor (Kawai & Kudo 2009).

Among the proposed functions of anther appendages in buzz-pollinated flowers, several could be relevant to Ericaceae, including attracting pollinators, influencing pollen release during buzzing or nectar feeding, and facilitating secondary pollen transfer. In this study, we tested two of these hypotheses: that anther spurs influence pollen release by triggering pollen expulsion during nectar feeding (hypothesis 3), and that they enhance pollen release in response to bee-generated vibrations (hypothesis 2). Although these functions have been hypothesized for Ericaceae, they have not been experimentally evaluated. Focusing on bilberry (*Vaccinium myrtillus* L.), we predicted, following the obstacle hypothesis (Müller 1873; Judd 1979; Dorr 1981), that flowers with intact anther spurs would exhibit increased pollen release compared to flowers from which the spurs had been removed when visited by nectar-feeding bees. Additionally, based on findings in *Huberia bradeana*, we hypothesized that the presence of spurs would similarly enhance pollen release during buzzing (Bochorny et al. 2021). To test these hypotheses, we quantified pollen removal from flowers with either intact or experimentally removed spurs in standardized array experiments. In the first experiment, flowers were visited by *Bombus terrestris* individuals, which were free to buzz during visitation. Recognizing that bumblebees can modulate some of their vibrational characteristics, such as buzz duration, to increase pollen release (Buchmann 1989; Kawai & Kudo 2009; De Luca & Vallejo-Marín 2013), a second experiment was conducted using a mechanical shaker to apply standardized vibrations to the flowers. Finally, to quantify the extent of natural variation in the trait under investigation, relative spur length was measured in flowers collected from ten different locations.

MATERIALS AND METHODS

STUDY SYSTEM

The flowers of *Vaccinium myrtillus* (bilberry or European blueberry) have poricidal anthers with spurs, and they produce nectar at the base of the flower (Fig. 2A). The corolla forms a bell-shaped structure (urceolate corolla) around the anthers that is orientated downwards with the stigma protruding outside of the corolla opening (Müller 1873, p.355). *V. myrtillus* is visited for both its nectar and its pollen, where the nectar visitors stick their head into the flower (Fig. 2B) and the buzzing visitors position their body around the bell and buzz (Fig. 2C).

PLANTS

To obtain bilberry flowers for the experiments, we clipped mature branches at the base of the aboveground part of the plant, resulting in 20 – 30 cm long plant cuttings. The plant cuttings were collected after their winter dormancy and before they started flowering (late April - early June). Approximately 10 plant cuttings were collected once a week from a population of *V. myrtillus* at Stadsskogen Uppsala, Sweden (59.846735, 17.622346) from March to June 2024 for the bee experiment and in February 2025 for the mechanical buzzing experiment. Cuttings were transported to a pollinator-free growth room with a photoperiod between 06:00-22:00, Daytime temperature 20°C, nighttime temperature 15°C, and light intensity: 222 micro-Einstein per square meter per second (μmol/m^2/s) PPFD. Cuttings were kept in these conditions for 7 – 14 days to

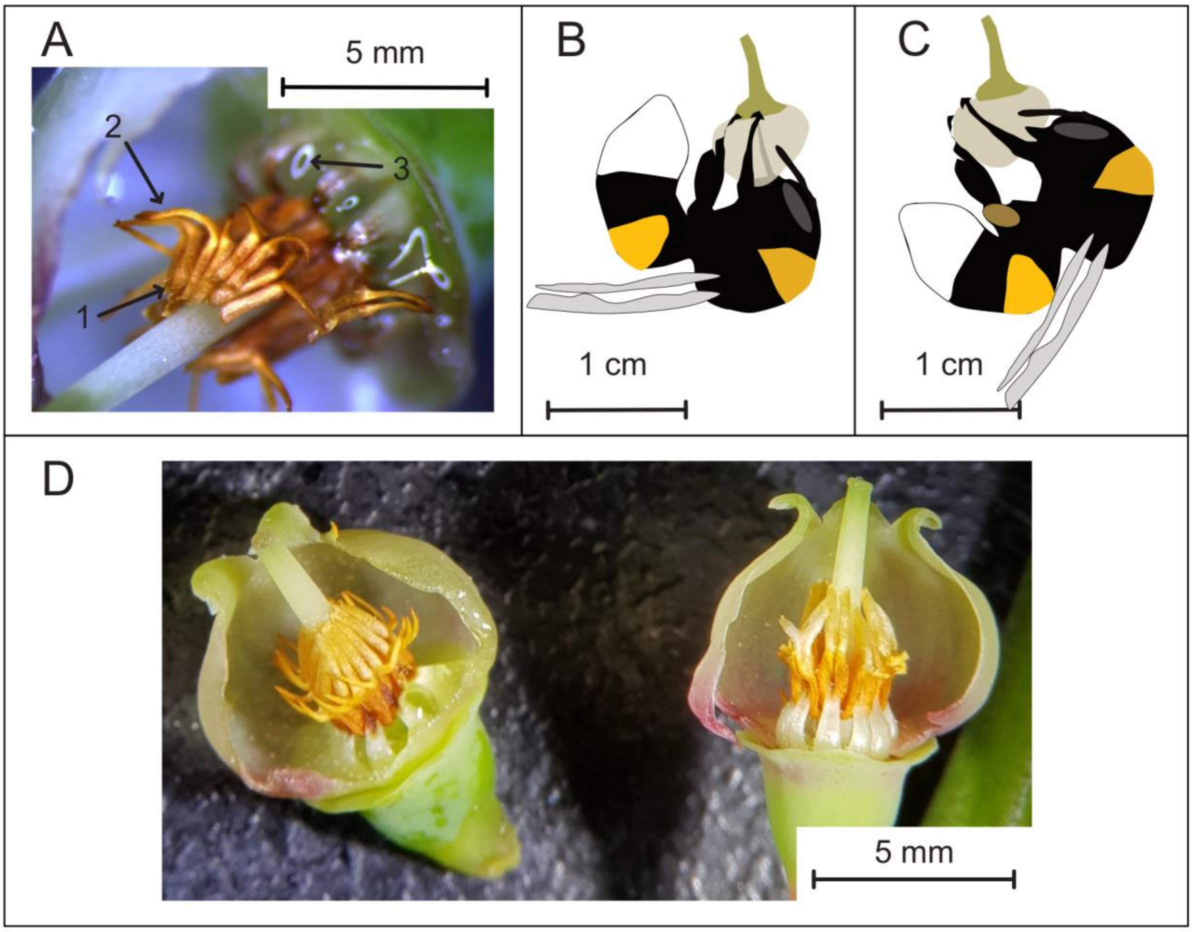

Figure 2. *Vaccinium myrtillus* flower morphology, bee-handling, and experimental manipulation done in the experiments. (A) Close-up image of a dissected flower of *V. myrtillus* showing (1) the pore of the poricidal anther where the pollen will be released, (2) the spur attached to the poricidal anther, and (3) the nectar gland. When the bee drinks from the nectar (Fig. 2B), the tongue is inserted through the small opening in the corolla and passes the spur downwards to the nectar gland. (B) *Bombus terrestris* worker drinking nectar from a *V. myrtillus*, notice the proboscis insertion in the flower opening. (C) *B. terrestris* worker buzzing on the flower of *V. myrtillus*. The bee bites into the corolla while curling her body around the opening, where the pollen will be released. (D) Unmanipulated flower on the left side, manipulated flower on the right side (one of the anthers in the manipulated flower has been moved out of position). The corolla was partly removed for illustration.

stimulate flowering. To delay the flowering of the plants we collected in the end of the natural growing season (in early June) we kept the plants in a cold room with conditions mimicking local autumn/winter conditions (photoperiod between 08:00-15:00, Daytime temperature 6°C, nighttime temperature 6°C, light intensity: 50 micro-Einstein per square meter per second (μmol/m²/s) PPFD) before transferring them to the growth room.

BEES

The flower visitor used in the experiment was *Bombus terrestris* from one lab-based colony (Biobest group, Belgium). This species is part of the pollinator community of local bilberries (*LvK unpublished field observation*; Bartholomée et al. 2024). The colony was approximately one month old and had unlimited access to a sugar solution provided by the supplier. In addition to the bilberry pollen that was collected during the experiment, the colony was given additional (honeybee-collected) pollen twice a week. The experiment was performed in a flight arena (see Appendix I for details).

BEE VISITING EXPERIMENT

To estimate the effect of the spur on pollen release, we tested the effect of bumblebees visiting flowers with spur and flowers where most of the spur was removed using micro-surgical scissors (Fig. 2D). To control for the effect of touching the flower during spur removal, the anthers and spurs of the unmanipulated flowers were also touched for a few seconds, mimicking cutting vibrations caused by the micro-surgical scissors, but without opening the scissors or actual cutting. The bumblebee colony was connected to the flight arena (Appendix I, Fig. A1). The bees were given three days with abundant bilberry flowers in the flight cage to get acquainted with them. During the experiment, a single bumblebee at a time was presented with 10 single-flower twigs in a water-filled tube with flower foam (Appendix I, Fig. A1). Bees were given up to 20 minutes to start visiting flowers before they were replaced by another bee. Whenever a flower was visited, we removed it from the flight arena to avoid double visits. The flowers that were in the arena together were all of the same treatment (Spur or No-Spur), and treatment for each bee was decided randomly using a coin toss. After a maximum of 10 flower visits, the bee was marked and returned to the colony. When, on another day, the same individual bee entered the flight arena again, the other treatment was done. During the experiment, we measured the time the bee spent on the flower, and we recorded whether the visit included the insertion of the proboscis, indicating nectar foraging and or buzzing of the flower. Because we did not measure any actual nectar consumption and buzzing bees often also insert their proboscis briefly into the flower when positioning themselves, we distinguish two visit types: buzzing vs. non-buzzing visits. After the visit, the flowers were stored in 500 μL of 70% ethanol. To estimate how much pollen the bees removed during their visits, the amount of pollen left in a visited flower was compared with the amount of pollen left in unvisited flowers. To control for individual variation between plants, we roughly alternated taking visited and unvisited flowers from the plants' branches, as well as the spur and no-spur treatment.

MECHANICAL BUZZING EXPERIMENT

To estimate the effect of the spur on pollen release during short, artificial flower visits with a standardized buzz, we tested 45 flowers with a spur and 40 without a spur. We chose the following properties of the buzz that mimic the buzz bees produce during a typical buzzing visit (Vallejo-Marín et al. 2022), and to make the results comparable with other studies using the same artificial buzzes. Vibrations were synthesized in Audacity (www.audacityteam.org) and applied using a mechanical shaker (Brüel & Kjær Permanent magnet Shaker LDS V201) connected to an audio amplifier (Brüel & Kjær linear power amplifier LDS LPA100). The signal consisted of four consecutive 250 ms buzzes (total duration: one second) at a frequency of 300 Hz and an amplitude of 250 m/s^2 (peak acceleration). The vibration was played on a computer connected via an amplifier to a vertically mounted shaker with an up-ramped, sideways orientation, resulting in horizontally propagated vibrations (Appendix II). A metal rod (size 4M) was attached to the shaker, with a 10 mm long enamelled insect pin (size 0) glued to its tip. Flowers were positioned using a clamp that held the twig just above the base of the corolla, and the pin was inserted into the upper half of the corolla to transfer the vibration. To prevent slippage during vibration, a small amount of honeybee wax was applied at the contact point (see Appendix II). This mode of attachment mimics how and where a bumblebee typically bites the corolla during pollination (see Fig. 2C). To calculate the proportion of pollen removed during a buzz, we needed to know the total number of pollen grains the flower contained before the buzzing. We therefore not only estimated the pollen left in the flower after a buzz, but we also collected the pollen released during the buzz in a 0.5 mL microcentrifuge tube held beneath the corolla opening. After each trial, both the flower with the remaining pollen and the collected pollen in the tube were preserved in 500 μL of 70% ethanol.

POLLEN COUNTING

The amount of pollen left in the flower was measured using a particle counter (Multisizer 4e Beckman Coulter Counter, Solna, Sweden). To make sure all the pollen was removed from the anthers and diluted in the ethanol, all tubes containing flowers were placed in a sonication bath 3 times for 3 minutes each. We then took 400 μm of vortexed ethanol solution containing the pollen, suspended this in 10 mL isotonic solution (Isoton II), and mixed it by pipetting to ensure a homogeneous distribution. Measurements were performed using a 100 μm aperture tube, and for each sample, 2000 μL of suspension was analysed. Only particles between 20 and 50 μm were analysed, thereby removing background noise and non-pollen debris. Both pollen size distribution and total grain count per sample were extracted using the Multisizer 4e software and used for subsequent analysis. The reported pollen size of *Vaccinium myrtillus* ranges between 37.0-50.5 μm, with a mean of 43.6 μm (Beug, 2004). However, based on the observed average and distribution of pollen grain sizes, total pollen counts per sample were estimated by summing all particles within the 30–40 μm size range.

NATURAL SPUR LENGTH VARIATION

To see if there is natural variation in spur length between different individuals of bilberries, we collected flowers from 10 different locations that are geographically so far apart that they could not possibly be the same individual (Appendix III Fig. A3). We measured 10 different flowers from different twigs per location and dissected 3 anthers per flower. All flowers were stored in 70% ethanol until dissection. Photos of the anthers were taken with a camera phone through the ocular of a dissecting microscope (Leica S9D; 25-35x magnification), and both the length of the anther (which we used as a proxy to capture variation in flower size) and the length of the spur from the base to the tip were measured using ImageJ (Schneider et al. 2012).

STATISTICAL ANALYSIS

To investigate the natural variation of relative spur length in natural conditions, we compared the ten locations using a linear mixed-effects model (LMM) (lme4 package; Bates et al. 2015). We modelled the average spur proportion as the response variable, with location as a fixed effect and individual flower included as a random effect to account for repeated measurements. Significance testing was done using a Type 3 ANOVA (lmerTest package; Kuznetsova et al. 2017). Afterwards, we compared the locations with each other in a pairwise comparison using estimated marginal means (emmeans package; Lenth 2024).

Bee experiment

To analyse the effect of spur and the effect of foraging behaviour (buzzing or non-buzzing), and the time spent on the flower on pollen removal in the bee experiment, we applied a LMM. In this analysis, we used the proportion of pollen removed as a response variable. Estimating pollen removed by the bee requires summing up the pollen that is collected on the bee's body including the corbiculae, pollen that falls to the ground, pollen that falls on the flower, and pollen transferred to other stigmas, and is thus a very hard quantity to observe directly (Minnaar & Anderson 2019; Vasquez-Castro et al. 2025). For this reason, we approximated pollen removal from the amount of pollen remaining in the anthers after visitation. The proportion of pollen removed in the bee experiment was calculated as follows:

$$\mathrm{Pr}_1 = 1 - \frac{\text{pollen in flower after visit}}{\text{mean pollen in unvisited flowers}}.$$

Since there was no significant difference between unvisited manipulated and unmanipulated flowers ($W = 624.5$, $P = 0.176$, see Appendix IV), we used both treatments when calculating the mean pollen in unvisited flowers. Pollen production per flower varies strongly even between flowers of the same individual (Vallejo-Marin & Lundgren, 2025, F. Gonzalez-Almansa, et al., unpublished) and thus, this way of calculating the proportion of pollen removed from the flower (Pr) often results in negative pollen removal when flowers have more pollen left after visitation than the mean number of unvisited flowers. To facilitate analysis and interpretation, we created and adjusted estimate of pollen removal ($\mathrm{Pr}_{\mathrm{norm}}$; range: 0 – 1) by calculating:

$$\mathrm{Pr}_{\mathrm{shifted}} = \mathrm{Pr}_1 - \mathrm{Pr}_{\mathrm{min}},$$

and normalizing it by the maximum value of $\mathrm{Pr}_{\mathrm{shifted}}$: $\mathrm{Pr}_{\mathrm{norm}} = \frac{\mathrm{Pr}_{\mathrm{shifted}}}{\max(\mathrm{Pr}_{\mathrm{shifted}})}$.

In the LMM, the fixed effects included Treatment (Spur vs. No spur), Behaviour (Buzz vs. No-Buzz), and Time on the flower (continuous) as well as their two-way and three-way interactions. Bee individual was included as a random intercept to account for repeated measures. Given that the response variable is proportional data, we applied an arcsine square root transformation to improve normality and homoscedasticity:

$$\mathrm{Pr}_{\mathrm{transformed}} = \arcsin\big(\mathrm{sqrt}(\mathrm{Pr}_{\mathrm{norm}})\big),$$

Significance testing was done using a Type 3 ANOVA. Model assumptions were assessed using the DHARMa package (Hartig 2016), testing residual uniformity (Kolmogorov-Smirnov test), outliers (binomial test), and dispersion (nonparametric dispersion test). No significant violations of dispersion were detected. A slight deviation from uniformity and a significant zero-inflation test result (driven by one exact zero) were observed but are unlikely to substantially affect inference (Zuur et al. 2009; Hartig 2016). To further explore interaction effects, we used the emmeans package in R to calculate estimated marginal means and pairwise contrasts between treatment and behaviour combinations, averaging over the continuous covariate (time on flower). Data visualization was done using sjPlot package (Lüdecke 2023).

Finally, to test the effect of time spent on the flower and its interaction with bee behaviour (Buzz vs. No-buzz) on the amount of pollen left after a visit, we fitted a separate LMM on the bee visiting experiment data, using the same general model structure and transformation approach described above. In this analysis, we were specifically interested in the effect of bee behaviour independent of spur removal.

Shaker experiment

In this experiment, we were able to directly estimate the proportion of pollen removed because we had both the remaining pollen in the flower and the pollen expelled with the shaker (collected in a microcentrifuge tube). The proportion of pollen removed in the mechanical buzzing experiment was calculated as follows:

$$\mathrm{Pr} = \frac{(\text{pollen in flower} + \text{pollen in tube}) - \text{pollen in flower}}{(\text{pollen in flower} + \text{pollen in tube})}.$$

Data were analysed using a generalized linear mixed model with a quasibinomial error distribution to account for overdispersion (dispersion parameter = 6455) observed in the pollen release proportions. Model diagnostics using DHARMa indicated minor deviations from ideal assumptions, including adjusted quantile deviations, but overall dispersion and outlier tests suggested the model fit was adequate for detecting treatment effects. All statistical analyses were performed using R version 4.1.0 (R Core Team, 2021).

RESULTS

BEE VISITS

In total, 83 manipulated (no spur) flowers and 112 unmanipulated (with spur) flowers were visited by 20 bumblebees, of which 7 individuals performed both a trial with "spur" flowers and a trial with "no spur" flowers. One bee performed the "no spur" trial twice because of a marking mistake. One bee attempted nectar robbing, a behaviour known from *Bombus terrestris*. To calculate the proportion of pollen removed in the bee experiment, we used 78 unvisited flowers (39 manipulated and 39 unmanipulated flowers). Because typical visits contained both an insertion of the proboscis into the flower (often short) and a buzz, where the bee bit the corolla while her body was curled around the opening, we could not isolate the effect of a buzzing visit from a combined visit. By far, most bees in our experiment buzzed when visiting a flower (176 out of 195 flower visits). In six trials, bees made at least one visit without buzzing (only nectar foraging behaviour), with the proportion of non-buzzing visits per trial ranging from 22.22% to 100% ($N = 19$ flower visits in 6 trials). Buzzing bees removed more pollen (60% ± 29%) than bees that did not buzz (23% ± 43%) (Fig. 3A; Table 2). The number of flowers visited by bees in trials with different treatments did not differ significantly ($W = 102$, $P = 0.564$), but buzzing visits were three times shorter than visits without a buzz (buzzing 21.70 s ± 24 s S.D., non-buzzing 63.79 s ± 70.45 s S.D.; $F_{1,76.29} = 13.16$, $P < 0.001$). The total range of time spent on the flowers in the bee experiment was between 3 and 267 s, and the average time was 26 s (± 34 s).

Spur effect

The mixed effects model, which included Treatment (Spur vs. No spur), Behaviour (Buzz vs. No-Buzz), Time on flower (continuous), and their two-way and three-way interactions, revealed significant effects of all predictors on the proportion of pollen removed by bees (Table 1, Appendix V and VI). A significant three-way interaction ($F_{1,145.96} = 13.96$, $P < 0.001$) indicates that pollen release depends on a combined influence of spur presence, bee behaviour, and visit duration. When averaging across both behaviours and all visit durations, flowers without a spur showed a slightly higher pollen removal (60% ± 30% S.D) than flowers with a spur (54% ± 33% S.D; Fig. 3B). Pairwise contrasts, averaged over visit durations, showed that buzzing visits removed significantly more pollen than non-buzzing visits in both spur (estimate = 0.23, $P = 0.031$) and no-spur treatments

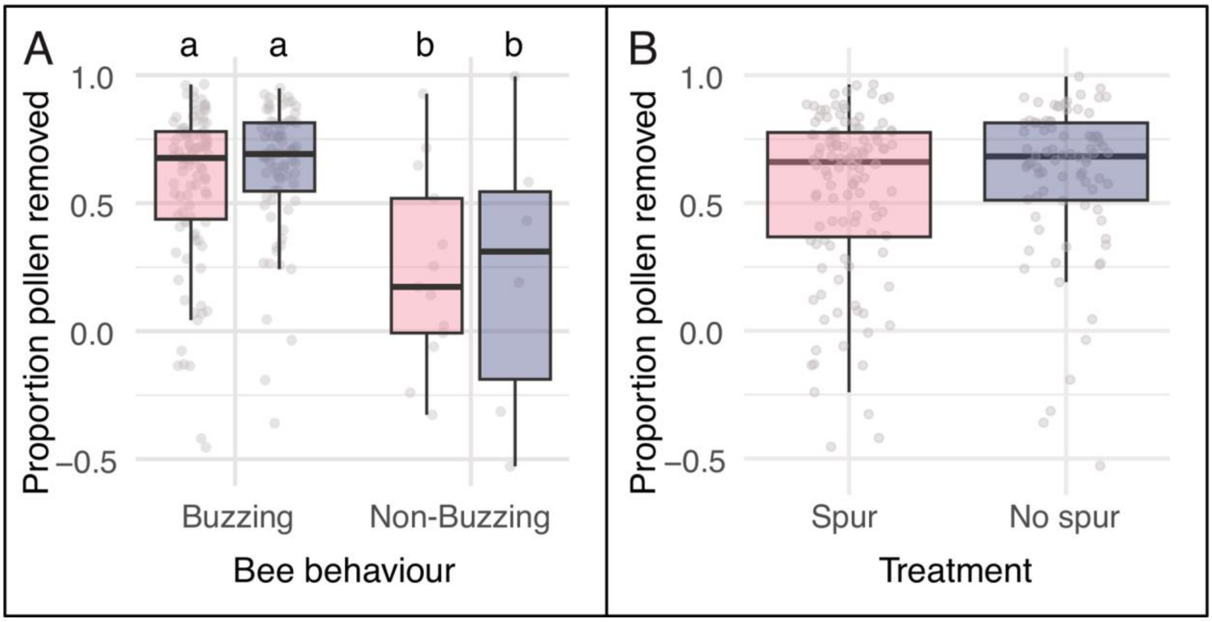

Figure 3. Panel figure showing the results of the bee experiment. (A) Effect of bee behaviour on pollen removal, pooled across visit durations. Buzzing visits removed significantly more pollen than non-buzzing visits in both treatments. Different letters (a, b) indicate statistically significant differences. (B) Effect of spur presence on pollen removal, averaged across behaviours and visit durations. Boxplots show the median and the spread of the untransformed data points. Proportion of pollen removal is calculated as: $\mathbf{Pr} = \mathbf{1} - \frac{\textbf{pollen in flower after visit}}{\textbf{mean pollen in unvisited flowers}}$. Negative values indicate instances where visited flowers contained more pollen than the average unvisited flower.

Table 1: Analysis of the proportion of pollen removed in the bee visiting experiment. Sample sizes per behaviour and treatment are: buzzing: spur *N* = 99, buzzing: no spur *N* = 77, non-buzzing: spur *N* = 13, and non-buzzing: no spur *N* = 6. Asterisk in the p-value column illustrates the significance level.

Effect	Estimate	Std. Error	F value	*P*-value
(Intercept)	0.996	0.040		
Treatment (Spur vs No spur)	0.096	0.052	4.13	0.0455 *
Behaviour (Buzzing vs Non-Buzzing)	-0.178	0.098	22.16	< 0.0001 ***
Time on flower	0.001	0.001	11.98	0.0007 ***
Time on flower : Behaviour	-0.002	0.001	7.58	0.0066 **
Time on flower : Treatment	-0.002	0.002	8.89	0.0033 **
Behaviour : Treatment	-0.608	0.207	8.63	0.0043 **
Time on flower : Behaviour : Treatment	0.015	0.004	13.96	0.0003 ***

(estimate = 0.46, *P* = 0.003). In contrast, spur presence did not significantly affect pollen removal within either behaviour type (*P* > 0.5; Table 2).

In the shaker experiment, significantly more pollen was released in flowers with no spur (28% ± 20% S.D) than in flowers with a spur (18% ± 15% S.D; Estimate = 0.67 ± 0.22, *t* = 2.99, *P* = 0.0036; Fig. 4). The average number of pollen grains in an unvisited, not vibrated flower in the bee experiment was 25,817 (± 11,305 S.D.) and in the artificial buzzing experiment was 36,703 (± 8,051 S.D.). The average size of a pollen grain was 33.51 µm (± 0.903 S.D, *N* = 163 785 pollen grains).

NATURAL VARIATION IN SPUR LENGTH

We found that the spur proportions of the flowers from the different locations differed significantly from each other ($F_{9,90} = 6.12$, $P < 0.001$). For three of the ten locations, the coefficients differed significantly from zero, indicating that the

Table 2: Estimated marginal mean contrasts for the proportion of pollen removed (arcsin-squared root transformed), extracted from the mixed model. All estimates are averaged over the observed distribution of visit durations (Time on flower). Asterisk in the p-value column illustrates the significance level.

Contrast	Estimate	Std. Error	95% CI	t ratio
Spur vs. No spur (Buzzing)	-0.0571	0.041	[-0.16,0.05]	-1.399
Spur vs. No spur (Non-buzzing)	0.178	0.145	[-0.20,0.56]	1.228
Buzzing vs. Non-buzzing (Spur)	0.227	0.082	[0.01,0.44]	2.772
Buzzing vs. Non-buzzing (No spur)	0.462	0.125	[0.13,0.80]	3.689

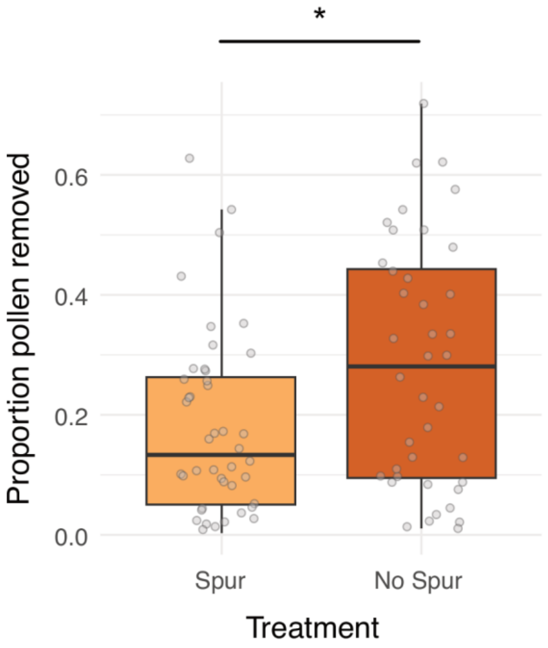

Figure 4. Proportion of pollen removed in flowers with and without a spur in the shaker experiment. Flowers with a spur released significantly less pollen than flowers without a spur (Estimate = 0.67 ± 0.22, t = 2.99, P = 0.0036). Boxplots show the median and the spread of the untransformed data points.

spur proportion varied notably between the different locations. The random effect of flower explained approximately 51% of the total variation, indicating that variation between individual flowers contributed substantially to the observed differences, while the remaining variation occurred within flowers. Pairwise comparisons confirmed significant differences in spur proportion between several locations. These differences are visualized in a boxplot (Appendix III Fig. A4), where locations with significantly different mean values are indicated.

DISCUSSION

ANTHER SPUR LIMITING POLLEN RELEASE

In two experiments, we tested the pollen release in flowers of *Vaccinium myrtillus* with and without a spur and found that flowers with a spur release less pollen than flowers without a spur. The effect of the flower spur was most apparent in the artificial buzzing experiment, where the effect of spur manipulation could be disentangled from bee behaviour. Our results seem to support the hypothesis that the anther spur could act as a flower mechanism to limit pollen release (Lloyd & Yates 1982; Harder & Thomson 1989; Johnson & Harder 2023). Such mechanisms exemplify a broader trend in floral evolution where plants develop precise control over pollen presentation in response to pollinator behaviour, thereby optimizing male fitness through staggered pollen release (Harder & Thomson 1989; Harder & Wilson 1994; Castellanos et al. 2006). From an adaptive perspective, pollen dosing can provide plants like *Vaccinium myrtillus* with a valuable strategy for maximizing reproductive success (Zhou et al. 2024).

Our bee experiment demonstrates that the effect of the spur was influenced by both bee behaviour (buzzing vs. non-buzzing) and visit duration. Visit duration appears to influence pollen release in non-buzzing visits to flowers without a spur (Appendix VI). While being cautious with any conclusions we draw from this small dataset (N = 19), it could be that flowers without a spur release less pollen than flowers with a spur in a short visit, but this effect reverses during long visits. An explanation for this could be that with the first tongue insertion, the spur could function as an obstacle (Müller 1883, p.377; Judd 1979; Dorr 1981), inducing pollen release, combined with the pollen limiting effect it might have during longer visits (similar to the buzzing visits), where more vibrations have been caused by the bee moving around on the flower. In addition to limiting pollen release and forming an obstacle, the anther spur in *V. myrtillus* could act as a site for secondary pollen transfer (Kawai & Kudo 2009). This kind of multifunctionality is common in floral traits and might enhance reproductive success through multiple interacting mechanisms. To understand what happens during non-buzzing (nectar visits), more data should be collected. Additionally, it would be interesting to evaluate the effect of the spur on pollen transfer success, as this might be a more relevant scale from the plant's perspective. To do this, one would need to work at a finer scale by experimenting with bees that do not buzz (such as male bumblebees or honeybees) and by tracking individual pollen grains, using, for instance, quantum dot labelling techniques (Kern et al. 2023).

The mechanisms behind the spur presence reducing pollen release are currently unknown. One possibility could be the added mass of the spur to the anther, thereby reducing the maximum acceleration that a bee can impart on the flower (Vallejo-Marín 2022). The anther spur in *Vaccinium myrtillus* is solid (Hermann and Palser, 2000) and could therefore contribute a relatively high amount of mass to the small anthers. Moreover, the location of the anther spur in *V. myrtillus* at the centre of the flower (Fig. 1) could reduce the anther displacement, similar to the effect observed in *Solanum elaeagnifolium* in Jankauski et al. (2022). Testing this hypothesis would benefit from 3D modelling approaches that simulate the addition of a mass at the spur's specific position on a poricidal anther. Exploring these biomechanical consequences contributes to our understanding of how floral structures evolve in response to physical constraints and pollinator interactions (Conner & Sterling 1995; Harder 2019).

POLLEN REMOVAL RATES

During our experiments, a single visit lasting 25.79 ± 33.76 s removed 57% (32% S.D.) of the pollen present in the flower, a percentage that falls within the range reported by Moquet et al. (2017) for pollen removal under natural conditions, confirming the ecological relevance of this lab-based study. Our data indicate that *Vaccinium myrtillus* releases more than half of its pollen within a single buzzing visit (60% ± 29%). Given that the plant benefits from distributing its pollen over multiple visitors (Harder & Thomson 1989), this represents a relatively high proportion for one visit. In contrast, nectar visits resulted in much lower pollen release (23% ± 43%), suggesting that these visits might contribute to a more gradual pollen dispersal. Pollen release in buzz-pollinated species can vary considerably, with single visits removing 9% in *Solanum rostratum,* 20% in *Pedicularis chamissonis*, 37% in *Solanum dulcamara*, and up to 70% in *Solanum laciniatum* (King & Buchmann 1996; Kawai & Kudo 2009; Vasquez-Castro et al. 2025). These differences potentially reflect the plant adapting its pollen release schedule to the local pollinator abundances, which might be very different from those in the Swedish forest understory (Bartholomée et al. 2024).

ECOLOGICAL IMPLICATIONS

Bilberries in the field are visited by both buzzing and non-buzzing bee species, where the non-buzzing nectar foraging visits have been shown to contribute more to the pollination of the flowers (Moquet et al. 2017). Bartholomée et al. (2024) showed that Swedish bilberries are pollen-limited and rely on their pollinator community for seed set. In general, forest understories tend to have relatively low pollinator abundance (Ulyshen et al. 2023), so offering both nectar and pollen rewards may be a strategy to balance their reproductive investment. Non-buzzing, nectar-visiting species, releasing less pollen per visit, could help spread pollen over multiple visits, while buzzing pollinators ensure effective pollen release when visits are infrequent. The contrast in pollen release between buzzing and non-buzzing visitors illustrates how plants might develop multi-functional floral traits to mediate interactions with different pollinator guilds. In buzz-pollinated lineages, structures like spurs, heteranthery, or poricidal anthers have evolved repeatedly, suggesting strong selective pressure for refined pollen-dispensing mechanisms under variable pollinator availability (Buchmann 1983; Vallejo-Marín 2019; Harder & Johnson 2009).

CONCLUSION

This study brings us one step closer to understanding the function of the anther spur in *Vaccinium myrtillus*. Our results show that the spur reduces pollen release during a visit, likely contributing to the plant's pollen-dispensing schedule. However, the bees' behaviour and the visit duration also influenced the effect of the spur on pollen release, with buzzing visits generally releasing more pollen than non-buzzing visits. Given the importance of non-buzzing nectar foragers in bilberry pollination under natural conditions, future research should focus on quantifying pollen release during nectar visits and exploring potential additional roles of the spur, such as facilitating secondary pollen displacement. These studies will help clarify how floral morphology and pollinator assemblages interact to shape pollen dispersal strategies in buzz-pollinated plants.

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AUTHOR CONTRIBUTION

Conceptualisation: LvK & MVM; methodology: LvK & MVM; investigation: LvK; formal analysis LvK; visualisation: LvK; writing - original draft: LvK; writing – review and editing: LvK & MVM; supervision: MVM.

AI STATEMENT

During the preparation of this work, the principal author (corresponding author) used CHATGPT for retrieving suggestions and equations for common statistical approaches and coding, and Grammarly and CHATGPT to improve writing style and check grammar and spelling. After using these tools/services, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

DISCLOSURE STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

Data are available in Figshare: 10.6084/m9.figshare.29831195

APPENDICES

Additional supporting information may be found in the online version of this article:

Appendix I. Flight arena and flower setup
Appendix II. Experimental setup mechanical shaker
Appendix III. Spur length comparison
Appendix IV. Comparing the pollen amount in unvisited flowers between the two treatments
Appendix V. Experimental results: mean and SD table
Appendix VI. Visualisation of mixed effects model results

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