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ORIGINAL RESEARCH

Pollen morphology for successful pollination dependent on pollinator taxa in a generalist plant: relationship with foraging behavior

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Abstract

Pollen morphology varies at inter- and intraspecific levels. Its interaction with pollinator behavior and morphology determines the probability of successful pollination. We tested whether pollen morphology promoting successful pollination differs depending on pollinator taxa in a generalist shrub, *Weigela hortensis* (Caprifoliaceae). We identified flower visitors carrying pollen from anthers to stigmas and compared the spine length and diameter of the pollen grains they carried. We found that pollen on the bodies of bumble bees and hunch-back flies and the scopae of small bees (including andrenid bees) contributed to seed production. Pollen grains on the bodies of bumble bees had longer spines than those on the scopae of andrenid bees or the bodies of hunch-back flies. Pollen grains on the bodies of bumble bees and the scopae of andrenid bees had larger diameters than those on hunch-back flies. Bumble bees collected pollen grains with shorter spines and larger diameters on their corbiculae while andrenid bees collected pollen grains with shorter spines and intermediate diameters on their scopae. The differences in morphology of pollen carried by pollinators reflected the tendency of bees to collect pollen with specific morphology into corbiculae/scopae. Our findings suggest that pollen morphology has diversified to facilitate successful pollination by pollinating partners.

Keywords Grooming behavior · Pollination · Pollen morphology · Scopa · *Weigela hortensis*

Introduction

Pollen grains must undergo successful transfer from anthers to stigmas to ensure plant reproduction. In angiosperms, 87.5% of species rely on animals for pollination (Ollerton et al. 2011). Since there are multiple routes of pollen loss, such as pollen grooming and passive pollen loss during transport (Inouye et al. 1994; Minnaar et al. 2019), only a small fraction of pollen produced by anthers reaches stigmas (Harder and Thomson 1989; Rademaker et al. 1997). Thus, plant male reproductive success depends highly on effective pollen transfer by pollinating animals. As pollinating animals vary in behavior and morphology (Krauss et al. 2017; Roquer-Beni et al. 2020; Stavert et al. 2016; Thorp 2000), flowers have evolved to facilitate pollen transfer from anthers to stigmas via their pollinating partners (e.g., Anderson et al. 2014; Castellanos et al. 2006; Parker et al. 2018).

Pollen morphology is diverse at various taxonomic levels and often considered responsible for the success of animal pollination in the context of pollinator behavior and morphology. In its interaction with pollinator behavior, pollen morphology may affect the probability of pollen consumption by bees. If bees collect pollen from bodies to transport structures, it is being taken to be consumed by larvae. Pollen grooming is a major route of pollen loss in bee pollination (Holmquist et al. 2012; Minnaar et al. 2019; Thomson 1986). A few studies comparing pollen collectability by bees among multiple plant species suggest that pollen grains with long spines or large diameters are less likely to be groomed into pollen transport structures (Hao et al. 2020; Vaissière and Vinson 1994). In the interaction between pollen and pollinator morphology, pollen spines could influence the probability of anchoring pollen grains to pollinator hairs (Lynn et al. 2020) while pollen grain size could influence the surface

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area contacting the hairs and therefore the adhesion force (Amador et al. 2017). Since insect hairs can vary in length and density among taxa (Roquer-Beni et al. 2020; Stavert et al. 2016), the spine length or size of pollen grains that adhere well to pollinators could depend on insect taxa. Lynn et al. (2020) examined the effects of intraspecific variation in pollen morphology on pollen pickup from flowers by bumble bees and flies in *Taraxacum ceratophorum* (Asteraceae). They found that pollen grains picked up by flies were larger in diameter (but not in spine length or spacing) than those picked up by bumble bees.

Here, we hypothesize that pollen morphology facilitating successful pollination differs according to pollinator taxa that exhibit different foraging behavior and morphology. An opportunity to test this hypothesis is provided by quantitative variations in pollen morphology within a plant species with various pollinator taxa (i.e., a generalist plant species). Hasegawa et al. (2023) showed that the generalist shrub, *Weigela hortensis* (Caprifoliaceae) had intraspecific variations in morphology of spiny pollen grains, and the flowers were visited by various taxa of bees and flies that differed in foraging behavior. We used this system to test our hypothesis by (1) identifying flower visitors carrying pollen from anthers to stigmas and characterizing their foraging behavior and then (2) comparing quantitative morphological traits of the pollen grains carried by these pollinators. Based on the results, we discuss the impact of pollen morphology on success in the generalized pollination system of *W. hortensis*.

Materials and methods

Study species and sites

Weigela hortensis (Caprifoliaceae) is a deciduous shrub that occurs in mountainous areas in Japan. From May to June, it produces many pale rose, bell-shaped flowers with 25–40-mm long corolla tubes. Flowers are self-incompatible and typically last for 4–5 days (Suzuki and Ohashi 2014). Our preliminary survey showed that the number of ovules per flower was 69 ± 2.1 [mean ± standard error (s.e.), $n = 39$].

The three study sites lie at different altitudes on Mt. Izumigatake, northern Honshu, Japan: Yoshinodaira [38°23′15″ N, 140°43′00″ E, 518 m above sea level (a.s.l.)], Otaira (38°24′51″ N, 140°43′14″ E, 650 m a.s.l.), and Taiwa (38°25′24″ N, 140°42′35″ E, 812 m a.s.l.). On Mt. Izumigatake, *W. hortensis* flowers are mainly visited by bumble bees (*Bombus*, Apidae), small bees (including Andrenidae and Halictidae), hunch-back flies (*Oligoneura* spp., Acroceridae), and hoverflies (Syrphidae) (Hasegawa et al. 2023). There are variations in assemblages of flower visitors along the altitudinal gradient; small bees are the predominant visitors at low-altitude sites whereas hunch-back flies increase in relative abundance with increasing altitude (Hasegawa et al. 2023).

Pollination effectiveness of flower visitors

From 24 May to 22 June 2022, we performed a field experiment to examine whether flower visits by different taxa of insects resulted in seed production at Yoshinodaira and Taiwa. At each site, 21 *W. hortensis* plants were used in the experiment. On each of one to three branches of each plant, two to seven buds were selected and all anthers in the buds were removed using forceps. This emasculation prevented pollen import by visitors from being influenced by the presence of pollen grains produced by the receiving flower. The branches were then bagged to exclude insects until visit observations were made. After flowering of the emasculated buds, the bags were removed to allow insects to visit the flowers for about 60 min. During this period, insect visits to flowers on each branch were recorded using a digital video camera (HDR-CX420, HDR-CX680, Sony, Japan; GZ-R400, GZ-RX690, JVCKENWOOD, Japan). We marked the emasculated flowers and measured their corolla tube lengths. After 60 min of open pollination, the branches were bagged again until the corollas abscised. Visitor observations were conducted for 3 days at each site. In total, visitors to 155 flowers on 33 branches (of 21 plants) at Yoshinodaira and 137 flowers on 36 branches (of 21 plants) at Taiwa were recorded. After fruit maturation, the marked fruits were collected and all seeds in the fruits were counted.

From the video recordings, visitors that contacted stigmas of the emasculated flowers were counted and classified into the following four groups: bumble bees, small bees, hoverflies, and hunch-back flies. Small bees possibly included Andrenidae, Halictidae, and Apidae although their families were rarely identifiable on the video recordings. Female bumble bees and small bees have pollen transport structures on their hind legs called corbiculae and scopae, respectively (Thorp 1979). Bumble bee and small bee groups were subdivided according to whether the corbiculae or scopae contacted stigmas, as determined by the video recordings. Since the scopa of a small bee is located around the hind legs, we determined that the inside surfaces of scopae contacted stigmas when small bees climbed on the stigmas.

Morphology and conspecific percentages of pollen carried by insects

From 31 May to 11 June 2021, we sampled visitors to *W. hortensis* flowers at Otaira and Taiwa to compare morphology of *W. hortensis* pollen carried by visitors among pollinator taxa and among body parts within taxa. Bees and flies were captured immediately after leaving *W. hortensis* flowers using plastic vials or a sweep net. Insects captured using a sweep net were then placed into plastic vials. The insects held individually in vials were immediately chilled on ice, transported to the laboratory, preserved at −20 ℃ for one day, and dried with silica gel at room temperature for more than 1 month. The insects were sent for taxonomic identification by experts after the pollen studies described below.

Pollen was sampled from the dorsal thoraxes of both bees and flies and from the corbiculae/scopae on the tibiae of the bees' hind legs. The dorsal thorax of corbiculate bees is known as one of the "safe sites," where pollen is less likely to be groomed away than it is from other body parts (Koch et al. 2017; Tong and Huang 2018). Thus, pollen that attaches to the safe sites is likely to be successfully transferred to a receptive stigma while pollen groomed into the corbiculae is much less likely to pass on its genes. For analysis with scanning electron microscopy, pollen on each body part of an insect was gently removed with a conductive carbon double-sided tape (5 mm width, Nisshin-EM, Japan) affixed to a cylinder specimen mount (10 mm diameter, Nisshin-EM, Japan). Because corbicular pollen, mixed with regurgitated nectar (Michener 1999), clumped more firmly when dried, it was split in two using forceps, and the section was gently pressed on a double-sided tape. Pollen on specimen mounts was then coated with platinum on an ion sputter coater (E-1045, Hitachi High-Tech, Japan) and observed using a scanning electron microscope (S-3400N, Hitachi High-Tech, Japan) at 3.0 kV. For each specimen mount, approximately eight *W. hortensis* pollen grains were randomly selected and photographed at 1000×. Then, the diameters and spine lengths of pollen grains in the images (Fig. S1) were measured using a program developed in Mathematica 11.1 (Wolfram Research 2017). The diameter of a pollen grain was defined as the diameter of the inscribed circle inside its outline. The spine length was defined as the mean length of the five longest spines forming a part of the outline of the pollen grain. In addition, to compare pollen traits between pollen on insects and in flowers, we reanalyzed images of pollen grains sampled from flowers (Hasegawa et al. 2023). These images were collected in 2020 at the same sites used in this study. Pollen was sampled from five fresh flowers of each of ten individual plants at each site, and six to ten pollen grains from each flower were photographed (Hasegawa et al. 2023).

Pollen carried by insects captured on *W. hortensis* flowers originated not only from *W. hortensis* flowers but also from heterospecific flowers. The percentages of *W. hortensis* pollen grains were estimated as one of the factors influencing pollination efficiency of insect visitors. For each specimen mount, three or four images of pollen grains were taken at 100× or 200× using a scanning electron microscope to include as many pollen grains as possible regardless of donor species. Up to 50 pollen grains for each specimen mount were classified according to whether they were *W. hortensis* or heterospecific. For specimen mounts with sparse pollen, we counted all *W. hortensis* and heterospecific pollen grains without taking images.

Statistical analyses

All statistical models were developed in PyMC3, a Python probabilistic programming framework for Bayesian parameter estimation (Salvatier et al. 2016). In all Bayesian statistical analyses described below, the No-U-Turn Sampler was used to generate four Markov chain Monte Carlo (MCMC) chains, each with 10,000 iterations following a burn-in period of 10,000 iterations. The potential scale reduction factors (R-hat) were below 1.01 for all parameters, indicating convergence of the MCMC chains. We performed posterior predictive checks to check that data generated from the fitted model is compatible with the observed data. Variance inflation factors in the models with multiple predictors were at most 1.52, suggesting that there were no problems of multicollinearity (Dormann et al. 2013).

We ran multiple regressions with Bayesian generalized linear mixed models (GLMMs) to test for the effect of visits by different visitor groups on seed production. We developed separate models for each study site because the compositions of flower visitors were markedly different between the sites. We used the visit and seed data for flowers receiving at least one visit with a stigma contact. However, we excluded the data for flowers on branches that did not produce any fruits with seeds, as these branches may have lacked carbohydrates or other nutrients. As a result, the visit and seed data for 40 fruits (on 16 branches of 12 plants) at Yoshinodaira and 96 fruits (on 30 branches of 19 plants) at Taiwa were analyzed. The multiple regression models included the number of seeds per fruit as an outcome variable and the number of visits with stigma contacts by different visitor groups and standardized corolla tube length as predictors. For bees, visits with stigma contacts by bodies or by corbiculae/scopae were included as separate predictors. The models also included branches as a random factor to account for repeated measures. Although we observed insect visits to 1–3 branches on the same plants, plants were not held as a random factor because only one branch was used for 8 out of 12 plants at Yoshinodaira and 12 out of 19 plants at Taiwa. A negative binomial sampling distribution was used with a log link function.

To compare pollen traits among pollen sources, we developed Bayesian GLMMs that included pollen grain spine length or pollen grain diameter as an outcome variable and pollen sources and sampling sites as predictors. The regressions for pollen sampled from insects were performed with a single model that included individual insects as a random factor. The models for pollen from flowers included plants and flowers as random factors. For pairwise comparisons between pollen sources, posterior distributions of differences between the means of trait distributions were estimated for all pairs of pollen sources using the MCMC samples. A gamma sampling distribution was used for both pollen grain spine length and pollen grain diameter.

To test for the effects of pollen traits on the probability of pollen collection from the bodies to the corbiculae/scopae of bees, we ran separate multiple logistic regressions with Bayesian GLMMs for each bee group. We used only the pollen data where pollen was sampled from both the bodies and corbiculae/scopae of the same individual bees. The multiple regression models included whether pollen was collected or not as an outcome variable (1.0 for pollen collected into the corbiculae/scopae and 0.0 for pollen remaining on the bodies), standardized spine length and diameter as predictors and the interaction of these predictors. We also incorporated the quadratic terms of both spine length and diameter in the models to distinguish whether the collection probability was highest at an extreme or intermediate pollen trait. When the estimate of a quadratic term was negative, we estimated an intermediate pollen trait value achieving the highest collection probability using the MCMC samples. The regression models also included sampling sites as a predictor and individual bees as a random factor. A Bernoulli sampling distribution was used with a logit link function.

To compare *W. hortensis* pollen percentages among pollen sources, we developed a Bayesian GLM with pollen sources and sampling sites as predictors. A beta-binomial sampling distribution, which allowed us to account for overdispersed count data (Gelman and Hill 2007), was applied to the numbers of *W. hortensis* and heterospecific pollen grains with a logit link function. For pairwise comparisons between pollen sources, posterior distributions of differences between the *W. hortensis* pollen percentages were estimated for all pairs of pollen sources using the MCMC samples.

Results

Pollination effectiveness of flower visitors

For flowers visited by insects at least once during a 60-min pollination period, the number of seeds per fruit was 10 ± 2.7 (mean ± s.e., $n = 40$) at Yoshinodaira and 19 ± 1.7 ($n = 96$) at Taiwa. At Yoshinodaira, bumble bees ($n = 7$), small bees ($n = 143$), and hoverflies ($n = 4$) were observed visiting *W. hortensis* flowers (Table S1). During visits, the bodies of all three groups and the scopae of small bees contacted stigmas. In total 72 visits included stigma contacts, and the bodies of small bees contacted stigmas most frequently ($n = 32$, 44.4%), followed by the scopae of small bees ($n = 30$, 41.7%), the bodies of bumble bees ($n = 7$, 9.7%), the bodies of hoverflies ($n = 3$, 4.2%), and the corbiculae of bumble bees ($n = 0$, 0.0%). At Yoshinodaira, visits with stigma contacts by the scopae of small bees had a positive effect on seed production (Fig. 1a). The model-estimated median of the coefficient was 1.241, indicating that the number of seeds per fruit increased by exp (1.241) ≈ 3.46 times with a single visit including a stigma contact by the scopa of a small bee. At Taiwa, bumble bees ($n = 152$), small bees ($n = 260$), hunch-back flies ($n = 196$), and hoverflies ($n = 43$) were observed as visitors (Table S1). During visits, the bodies of all four groups and the corbiculae/scopae of the bees contacted stigmas. In total 459 visits included stigma contacts, and the bodies of bumble bees contacted stigmas most frequently ($n = 133$, 29.0%), followed by the bodies of hunch-back flies ($n = 124$, 27.0%), the bodies of small bees ($n = 78$, 17.0%), the scopae of small bees ($n = 77$, 16.8%), the bodies of hoverflies ($n = 35$, 7.6%), and the corbiculae of bumble bees ($n = 12$, 2.6%). At Taiwa, visits with stigma contacts by the bodies of bumble bees and hunch-back flies had positive effects on seed production (Fig. 1b). The model-estimated medians of the coefficients were 0.205 and 0.137, indicating

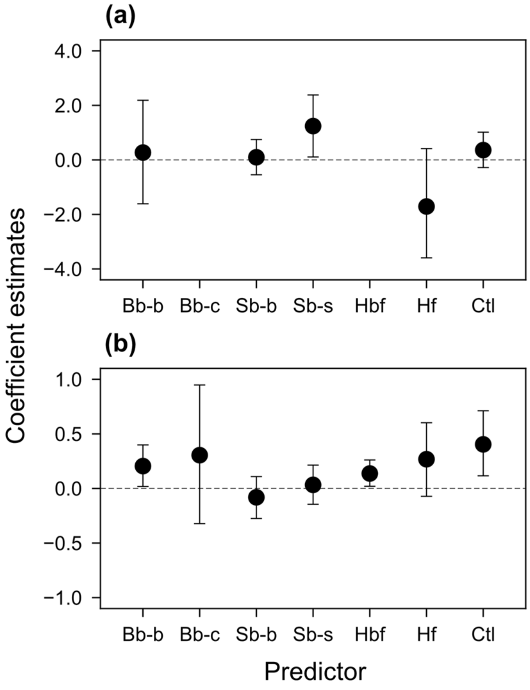

Fig. 1 Posterior coefficient estimates from Bayesian multiple regression models testing for the effect of visits to *Weigela hortensis* flowers by different visitor groups on seed production at **a** Yoshinodaira and **b** Taiwa. All estimated coefficients are shown as the medians (points) with the 95% highest density intervals (HDIs) (whiskers). A predictor does not have a significant effect on seed production if the 95% HDI includes zero. At Yoshinodaira, the corbiculae of bumble bees were not observed to contact stigmas. Predictors: the number of stigma contacts by bodies of bumble bees (Bb-b), corbiculae of bumble bees (Bb-c), bodies of small bees (Sb-b), scopae of small bees (Sb-s), bodies of hunch-back flies (Hbf), bodies of hoverflies (Hf), and corolla tube length of a visited flower (Ctl)

that the number of seeds per fruit increased by approximately 1.23 and 1.15 times with a single visit including a stigma contact by the body of a bumble bee or a hunch-back fly, respectively. At Taiwa, the number of seeds per fruit also increased with increased corolla tube length (Fig. 1b).

Morphology and conspecific percentages of pollen carried by insects

At Otaira and Taiwa, the following insects were collected (Table S2): bumble bees (*Bombus diversus*, *B. honshuensis*, *B. ardens*, and *B. hypocrite*, Apidae), andrenid bees (*Andrena lonicerae*, Andrenidae), halictid bees (*Lasioglossum* spp., Halictidae), and hunch-back flies (*Oligoneura nigroaenea*, Acroceridae). Of the bumble bees collected, 19 were females and three were males from Otaira, while six were females and seven were males from Taiwa. Corbicular pollen was found on 17 out of the 19 female bumble bees at Otaira and four out of the six female bumble bees at Taiwa. Scopal pollen was found on 22 out of 25 andrenid bees at Otaira and all 13 andrenid bees and three out of eight halictid bees at Taiwa. None of the seven halictid bees at Otaira had pollen on the tibial scopae. Due to small sample sizes, we did not conduct analyses involving corbicular pollen on bumble bees at Taiwa or pollen on halictid bees at either site.

Pollen grain spine length and pollen grain diameter were compared among the bodies of bumble bees, the scopae of andrenid bees, and the bodies of hunch-back flies, the visitor groups identified as effective in seed production in our field experiment (Fig. 1). The spine length of pollen grains on the bodies of bumble bees was greater than the spine lengths of pollen grains on the scopae of andrenid bees or the bodies of hunch-back flies (Fig. 2a; Table 1). Pollen grains on these pollinator groups had shorter spines than pollen grains in flowers (Fig. 2a). The diameters of pollen grains on the scopae of andrenid bees and the bodies of bumble bees were larger than the diameter of pollen grains on the bodies of hunch-back flies (Fig. 2b; Table 1). Pollen grains on the bodies of hunch-back flies were smaller than pollen grains in flowers (Fig. 2b). The spine length was greater in pollen grains sampled from pollinators at Otaira (the lower-altitude site) than at Taiwa. Sampling sites did not have significant effects on the diameter of pollen grains on pollinators or the spine length or diameter of pollen grains in flowers (Table 1). Pairwise comparisons including pollen sources that hardly contributed to seed production were also conducted; pollen grains on the bodies of andrenid bees had shorter spines than those on the bodies of bumble bees and longer spines and larger diameters than those on hunch-back flies (Fig. S2a, b and Table S3).

The probability that pollen was collected from the bodies to the corbiculae of bumble bees increased with decreasing spine length and increasing diameter (Fig. 3a, b; Table 2). The probability of pollen collection into the scopae of andrenid bees tended to increase with decreasing spine length, similar to bumble bees (Fig. 3c; Table 2). In addition, the quadratic term of pollen grain diameter was negative (Table 2), indicating that pollen with an intermediate diameter had the highest probability of being collected into the scopae (Fig. 3d). The model-estimated median of the diameter associated with the highest

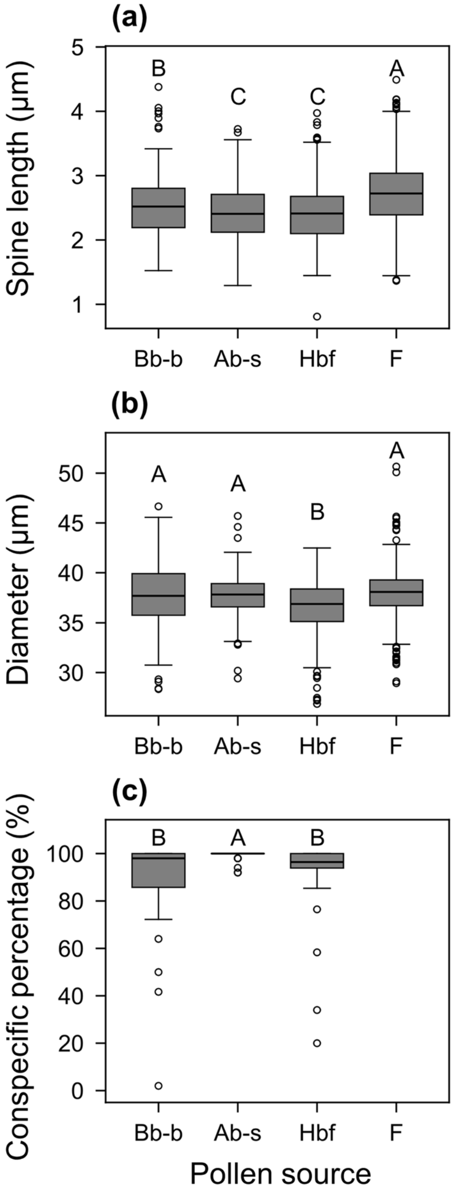

Fig. 2 **a** Spine length, **b** diameter, and **c** conspecific percentage of *Weigela hortensis* pollen grains sampled from flowers and the visitor groups identified as effective in seed production. In each panel, pollen sources sharing a letter were not significantly different, i.e., the 95% highest density interval (HDI) of the difference of the means derived from Bayesian models did not exclude zero. Pollen sources: (Bb-b) bodies of bumble bees, (Ab-s) scopae of andrenid bees, (Hbf) bodies of hunch-back flies, and (F) flowers

Table 1 Posterior coefficient estimates from Bayesian regression models comparing spine length, diameter and conspecific percentage of *Weigela hortensis* pollen grains among pollen sources and sampling sites

Outcome variable	Intercept/predictor	Coefficient estimate
Spine length (pollen on insects)	Intercept [bumble bee (body)/Otaira]	0.945 (0.910, 0.978)
	Andrenid bee (scopa)	−0.050 (−0.095, −0.006)
	Hunch-back fly	−0.044 (−0.083, −0.003)
	Taiwa	−0.048 (−0.081, −0.014)
Spine length (pollen in flowers)	Intercept (Otaira)	0.999 (0.920, 1.079)
	Taiwa	0.007 (−0.105, 0.120)
Diameter (pollen on insects)	Intercept [bumble bee (body)/Otaira]	3.628 (3.615, 3.640)
	Andrenid bee (scopa)	0.002 (−0.014, 0.018)
	Hunch-back fly	−0.028 (−0.042, −0.014)
	Taiwa	−0.001 (−0.013, 0.011)
Diameter (pollen in flowers)	Intercept (Otaira)	3.634 (3.615, 3.653)
	Taiwa	0.000 (−0.027, 0.028)
Conspecific percentage	Intercept [bumble bee (body)/Otaira]	2.584 (2.040, 3.137)
	Andrenid bee (scopa)	1.355 (0.591, 2.156)
	Hunch-back fly	0.027 (−0.524, 0.585)
	Taiwa	−0.634 (−1.140, −0.156)

All estimated coefficients are listed as the median with the 95% highest density interval (HDI) in parentheses. A predictor does not have a significant effect on an outcome variable if the 95% HDI includes zero. Spine length or diameter of pollen on insects and in flowers were regressed with separate models

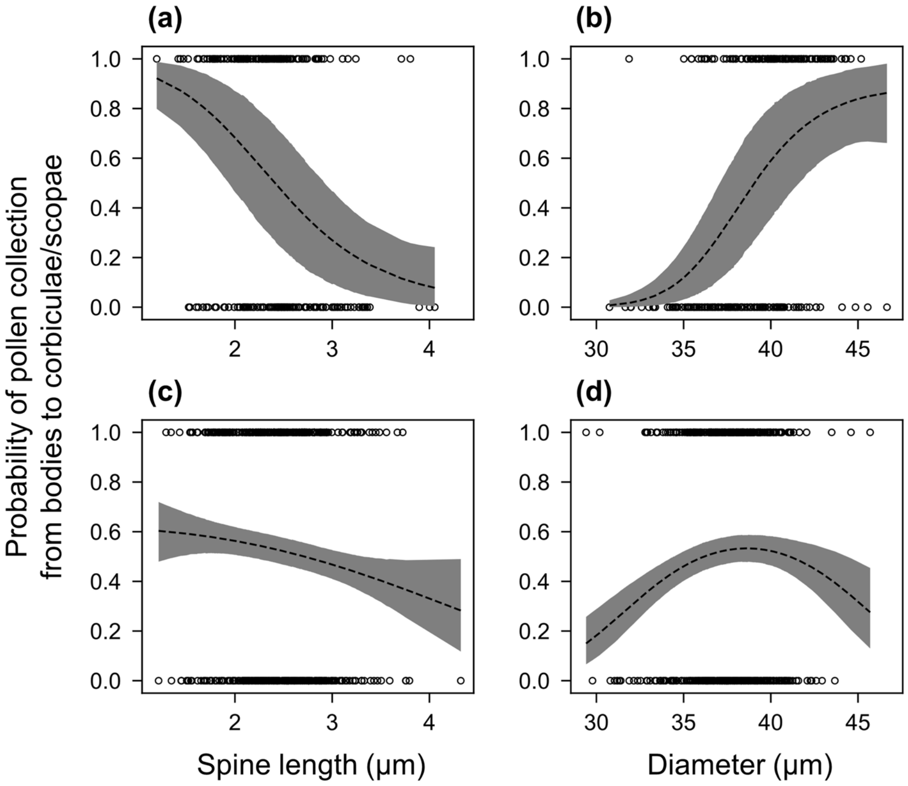

Fig. 3 The effects of pollen grain spine length and diameter on the probability of pollen collection from bodies to corbiculae/scopae of bees. **a**, **b** bumble bees, **c**, **d** andrenid bees. Multiple logistic regressions incorporating the quadratic terms of predictors were conducted. On the vertical axis, 1.0 represents pollen collected into corbiculae/scopae, and 0.0 represents pollen remaining on bodies. The medians (dashed lines) and the 95% highest density intervals (HDIs) of the collection probabilities were derived from Bayesian models that held the other predictor constant at their mean values and included individual insects as a random factor. Due to the small sample size, pollen on bumble bees at Taiwa was excluded from the regression analysis

collection probability was 38.63 μm [95% highest density interval (HDI): 37.54–40.21 μm], which was larger than the mean diameter of pollen grains on the bodies of andrenid bees (37.40 μm; Table S2). Sampling sites did not have a significant effect on the probability of pollen collection (Table 2).

Compared among the pollinator groups, the percentage of *W. hortensis* pollen was higher on the scopae of andrenid

Table 2 Posterior coefficient estimates from Bayesian multiple logistic regression models examining the effects of pollen grain spine length and diameter on the probability of pollen collection from bodies to corbiculae/scopae of bees

Pollen source	Predictor	Coefficient estimate
Bumble bee	Intercept	−0.004 (−0.465, 0.465)
	Spine length	−0.871 (−1.272, −0.492)
	Spine length^2	0.046 (−0.197, 0.280)
	Diameter	1.150 (0.784, 1.543)
	Diameter^2	−0.171 (−0.470, 0.110)
	Spine length × Diameter	−0.184 (−0.564, 0.223)
Andrenid bee	Intercept (Otaira)	0.106 (−0.154, 0.355)
	Spine length	−0.170 (−0.346, 0.006)
	Spine length^2	−0.020 (−0.136, 0.098)
	Diameter	0.105 (−0.082, 0.281)
	Diameter^2	−0.106 (−0.216, −0.001)
	Spine length × diameter	0.014 (−0.159, 0.194)
	Taiwa	0.099 (−0.258, 0.470)

All estimated coefficients are listed as the median with the 95% highest density interval (HDI) in parentheses. A predictor does not have a significant effect on the probability of pollen collection if the 95% HDI includes zero. Due to the small sample size, a regression analysis for bumble bees at Taiwa was not conducted

bees (99.14 ± 0.35%, mean ± s.e.) than on the bodies of bumble bees (88.25 ± 3.49%) or hunch-back flies (93.26 ± 1.81%) (Fig. 2c; Table 1). In addition, the percentage was higher in insects from Otaira than from Taiwa (Table 1). Comparisons of the *W. hortensis* pollen percentages relative to all pollen sources showed that the percentages on the bodies of andrenid bees were higher than on the bodies of bumble bees and hunch-back flies, while pollen on the corbiculae of bumble bees had the lowest percentage (Fig. S2c; Table S3).

Discussion

Pollination effectiveness of flower visitors

The field experiment on pollination effectiveness suggested that *W. hortensis* pollen was carried from anthers to stigmas by three taxa of pollinators: bumble bees, small bees, and hunch-back flies. Both pollinating bees shared the common behavior of actively collecting *W. hortensis* pollen into their transport structures, but they differed in transferring pollen attached to different body parts to stigmas. Bumble bees contributed to seed production by transferring pollen on bodies to receptive stigmas (Fig. 1b). Although pollen grains stored on corbiculae may also move to stigmas, they are sometimes impaired due to being moistened with nectar (Parker et al. 2015). Conversely, small bees, including andrenid bees and halictid bees, transferred scopal pollen to stigmas (Fig. 1a). Most andrenid bees captured on *W. hortensis* flowers carried a lot of pollen on their scopae, where pollen remains dry and loose without mixing with nectar, whereas only a few halictid bees carried pollen on their scopae. We also found that andrenid bees are faithful to *W. hortensis* flowers as the percentages of *W. hortensis* pollen on the scopae of andrenid bees were more than 98%, the highest among pollinator groups (Fig. 2c; Table S2). Pollination effectiveness of andrenid bees may be linked to their pollen grooming behavior and consistent visits to *W. hortensis* flowers.

Hunch-back flies are distinctly different from bees in terms of morphology and behavior; they have a lower level of hairiness than bees, feed on nectar but not pollen in *W. hortensis* flowers and do not groom body pollen (T.M. Hasegawa, personal observation). In addition, hunch-back flies were faithful to *W. hortensis* flowers. The *W. hortensis* pollen percentages on their bodies exceeded 90% on average even at the higher-altitude site, Taiwa (Fig. 2c; Table S1), where the overall *W. hortensis* pollen percentages were relatively lower compared with Otaira (Table 1), probably due to the greater diversity of flowering plant species. Comparison of the regression coefficients suggested that hunch-back flies and bumble bees were not greatly different in pollination efficiency (Fig. 1), reflecting the fact that hunch-back flies consistently foraged for nectar but not pollen of *W. hortensis* flowers. Hoverflies also visited flowers but were not effective in pollination (Fig. 1). They differed from hunch-back flies in their pollen predation (T.M. Hasegawa, personal observation). Pollination effectiveness should be influenced by foraging behavior of visiting insects.

Effective pollinators were different between study sites. Small bees were effective at the lower-altitude site, Yoshinodaira (Fig. 1a), where flowers were predominantly visited by small bees (Table S1). On the other hand, they had little effect on seed production at the higher-altitude site, Taiwa (Fig. 1b), where small bees, bumble bees, and hunch-back flies visited flowers at similar frequencies (Table S1). In contrast, bumble bees and hunch-back flies were effective (Fig. 1b), suggesting that they may be more efficient pollinators of *W. hortensis* than small bees.

Pollen grain spine length for successful pollination

The comparison analyses of morphology of pollen carried by different pollinator groups showed that the pollen morphology that promotes successful pollination depends on pollinator taxa. Pollen grains on the bodies of bumble bees had longer spines than pollen grains on the scopae of andrenid bees or the bodies of hunch-back flies (Fig. 2a). This suggests that pollination by bumble bees is facilitated by longer pollen spines than pollination by andrenid bees or hunch-back flies. The differences in spine length between them may reflect their different levels of hairiness, which could influence the degree of pollen grain anchoring (Thorp 1979) and a common tendency for bumble bees and andrenid bees to collect pollen grains with shorter spines (Fig. 3a, c). This tendency is consistent with the results of previous work that compared the collectability of pollen from multiple species for corbiculate bees (bumble bees or honey bees) (Lunau et al. 2015; Vaissière and Vinson 1994). For the first time, we provided evidence that the tendency to collect pollen grains with shorter spines into the corbiculae/scopae is common to corbiculate bees and non-corbiculate andrenid bees. Furthermore, this finding suggests that selection for spine lengths of pollen grains is at least partially due to effects on defense against pollen collection into bumble bee corbiculae or aid to pollen collection into andrenid bee scopae.

The spine lengths of pollen grains on insect bodies differed between the two sampling sites, although the spine lengths of pollen grains in flowers did not (Table 1). One possible explanation for this discrepancy could be the abundance of pollinators. In environments where flowers are frequently visited by pollinators, even pollen grains with morphologies that are less likely to be removed from anthers could be removed by pollinators. Therefore, in addition to pollinator taxa, pollinator abundance may influence the morphology of pollen grains adhering to pollinator bodies.

Weigela hortensis flowers produced pollen grains with spine lengths longer than those facilitating adhesion to pollinators (Fig. 2a). Pollen spines could also affect pollination processes beyond pollen adhesion to a pollinator, such as pollen transfer from a pollinator to a stigma. For example, in *Helianthus annuus* (Asteraceae), interlocking between pollen spines and stigma papillae enhances adhesion of a pollen grain to a stigma (Lin et al. 2016). Alternatively, pollen spines could accumulate electric charge and influence electrostatic forces between a pollen grain and a stigma (Inchaussandague et al. 2018). The effects of pollen spine phenotypes on the probability that pollen on a pollinator is transferred to and retained on a stigma should be examined in future studies.

Pollen grain diameter for successful pollination

Pollen grains on the bodies of bumble bees and the scopae of andrenid bees had larger diameters than pollen grains on the bodies of hunch-back flies (Fig. 2b), suggesting that pollination by bumble bees and andrenid bees could be facilitated by larger pollen compared with pollination by hunch-back flies. This may result from the different levels of hairiness between bees and hunch-back flies. The length and spacing of insect hairs could influence the sizes of pollen grains carried on the hairs (Amador et al. 2017). In addition, there was a tendency of andrenid bees to collect pollen with a slightly larger diameter than the mean diameter of body pollen (Fig. 3d). The pollen diameter giving a high probability of pollen collection by andrenid bees might ensure a mechanical fit between branched hairs of the scopae (Amador et al. 2017; Thorp 1979, 2000). In contrast, bumble bees collected larger pollen more easily (Fig. 3b). As Harder (1998) proposed, larger pollen could be more easily groomed with the comb-like structures on bees' legs. Although Harder (1998) did not find evidence supporting this hypothesis by comparing pollen grain size between plant species with pollinators that exhibit different grooming behavior, our survey provided evidence by directly examining the effect of intraspecific quantitative variation in pollen grain diameter on pollen collectability. This finding seems to disagree with the result of Hao et al. (2020) showing that pollen grain diameter was larger in plant species from which bees did not collect pollen than in plant species from which bees did collect pollen. However, as Hao et al. (2020) indicated, their result might reflect the possibility that selection is actually on pollen number, which negatively correlates with pollen size.

Pollen morphological variation in a generalist plant

The comparisons of morphology of pollen carried by pollinators suggested that different pollen grain spine lengths and pollen grain diameters could be selected for by different pollinator groups. Such selection may lead to adaptive differentiation in pollen morphology between populations with different pollinator assemblages.

On Mt. Izumigatake spine length of *W. hortensis* pollen grains was greater at higher-altitude sites, and small bees were the predominant visitors at low-altitude sites whereas hunch-back flies increased in relative abundance with increasing altitude (Hasegawa et al. 2023). Our finding that andrenid bees collected pollen grains with shorter spines on their scopae (Fig. 3c) supports the idea that the altitudinal variation in pollen grain spine length is the result of selection favoring shorter spines exerted by low-altitudinal pollinators. However, contrary to our expectation, the mean spine length of pollen grains collected on the scopae of andrenid bees was not significantly smaller than that of pollen on hunch-back flies (Fig. 2a). Therefore, we cannot confidently conclude that the altitudinal variation in spine length has resulted from the difference in local pollinator assemblages. Nevertheless, andrenid bees and hunch-back flies could exert different selection pressures on spine length. Theoretical work by Hasegawa et al. (2021) suggests that two different pollinators that optimally pollinate using pollen grains with the same spine length can select for different spine lengths given the cost of producing pollen spines. If one pollinator has stronger grooming intensity than the other, it would lead to a narrower range of effective spine lengths for pollination, thereby increasing the selection pressure on the length of costly spines. With such a mechanism, pollinators could select for different spine lengths and cause altitudinal variation.

In the present study, bumble bees visited flowers frequently and were identified as effective pollinators at the higher-altitude site, Taiwa (Fig. 1; Table S1). In contrast, Hasegawa et al. (2023) showed that the number of bumble bee visits per flower was relatively small and almost independent of altitude. Because the visit frequency of bumble bees seemed to have annual or daily fluctuations, bumble bees might not contribute to altitudinal variation in selection pressures on pollen morphology.

Whereas pollen grain diameter for successful transfer to pollinators depended on pollinator taxa (Fig. 2b), altitudinal variation in pollen grain diameter was not found in Hasegawa et al. (2023). Pollen grain size may influence reproductive success not only in the pollination process but also in post-pollination processes. During post-pollination processes, large pollen grains may outperform small ones in pollen competition for ovule fertilization (McCallum and Chang 2016). In that case, there would be an optimal pollen grain size maximizing success in post-pollination processes under pollen grain size–number trade-offs (Smith and Fretwell 1974; Vonhof and Harder 1995). Therefore, pollen grain size could be under stabilizing selection in post-pollination processes, and the pollen grain size optimal for the pollination process may not necessarily have been selected for. For a better understanding of pollen grain size evolution in *W. hortensis*, the effect of pollen grain size on post-pollination success should be examined in future studies.

Conclusions

We showed that *W. hortensis* pollen morphology that promotes successful pollination depends on pollinator taxa. The differences in pollen grain spine length and pollen grain diameter between pollinators from different taxa reflected the tendency of bees to collect pollen with specific morphology into corbiculae/scopae. Morphological traits of specific pollinators may also have had an effect. The results also suggest that selection for pollen morphology is at least partially due to its roles in defense against or aid to pollen collection into pollen transport structures by bees. This study extends the knowledge of interactions between pollen and pollinators and raises the possibility that pollen morphology has diversified to facilitate the success of pollination by a variety of pollinating partners. From another point of view, the complementarity of pollinators in terms of the morphology of pollen they carry suggests that variation in pollen morphology in a generalist plant may ensure reproductive success. Further studies that associate pollen morphology with pollen deposition on stigmas and post-pollination success will improve our understanding of the diversity of pollen morphology.

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Declarations

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