# Effect of pollinator size on seed set in *Lamium album* var. *barbatum*

## Mitsuru Hattori[1,*,‡], Yoko Tamada[2,‡] & Takao Itino[3, 4]

[1]Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki city, Nagasaki, Japan
[2]Graduate School of Science and Technology, Department of Science, Shinshu University, Matsumoto city, Nagano, Japan
[3]Department of Biology, Faculty of Science, Shinshu University, Matsumoto city, Nagano, Japan
[4]Institute of Mountain Science, Shinshu University, Matsumoto, Matsumoto city, Nagano, Japan
[‡]These authors contributed equally to the paper
*Corresponding author: mhattori@nagasaki-u.ac.jp

**Background and aims** – Previous researchers have demonstrated that geographic variation in pollinator community composition can generate diversity in the floral traits of animal-pollinated plants. Our study focused on the bumblebee-pollinated white dead-nettle *Lamium album* var. *barbatum*. Geographic variation in corolla length of this species is known to be correlated with regional pollinator size. The aim of this study is to clarify whether size-matching between flower and pollinator affects seed set in *L. album*.

**Material and methods** – In the present study, we investigated two *L. album* populations on Mount Norikura, central Japan. We determined the pollinator community composition and corolla length during the flowering period of *L. album* and recorded seed set after a single visit by different pollinator categories.

**Key results** – We observed that the main pollinators of *L. album* were bumblebee queens and workers. Bumblebee queens visited flowers more frequently than workers during peak flowering. Furthermore, size-matching between flowers and bumblebee queens, but not workers, strongly promoted seed set. These results suggest that *L. album* flower size is adapted to bumblebee queens, the main pollinator during peak flowering season in our study sites.

**Keywords** – Lamiaceae; pollination mutualism; pollinator mediated selection; size-matching.

## INTRODUCTION

To understand diversification in flowers, focusing on the relationship between the geographic variation in floral traits and that of pollinator community composition can provide important insights. Several previous studies have shown that geographic variation (e.g. altitudinal variation) in pollinator community composition leads to diversification of floral traits, such as flower size, through pollinator-mediated selection (Nagano et al. 2014; Kuriya et al. 2015). To understand the effect of pollinator-mediated selection on flower diversification, it is not only necessary to observe geographical correlation between flower size and pollinator size, but also to determine the effect of mechanical fit on plant fitness; however, several previous studies have observed only size correlations (e.g. Newman et al. 2015;

Hattori et al. 2015). In the present study, we observed the pollinators associated with *Lamium album* L. var. *barbatum* (Lamiaceae) and determined whether the size-match between functional nectar depth (i.e. flower length) and pollinator length (i.e. head length + proboscis length) affected the female fitness of the plant.

*Lamium album* var. *barbatum* is a perennial herb broadly distributed in East Asia (Hayashi 2009). It produces bi-lipped white-pink flowers that open from April to July. Corolla length significantly differs among populations in central Japan and the maximum difference between populations was around 25% of the whole flower length (29–36 mm; Hattori et al. 2015). Previous research has found that *L. album* is pollinated by four bumblebee species and other bees in central Japan (Hattori et al. 2015). Furthermore, although geographic variation in corolla length correlates

with pollinator size (Hattori et al. 2015), there is no proof yet that size matching between flowers and pollinators actually affects fitness of *L. album*. We hypothesized that the observed corolla length variation of *L. album* is an adaptation to local pollinator communities.

## MATERIAL AND METHODS

### Study sites

We focused on two populations of *L. album*, 1488 m a.s.l. (P1, 36°07′56.63″N, 137°37′52.84″E) and 1649 m a.s.l. (P2, 36°07′54.11″N, 137°37′52.41″E) on Mount Norikura with a summit of 3026 m a.s.l., Nagano, central Japan (36°07′29″N, 137°33′11″E). Both populations grew along edges of mixed forests consisting of deciduous broad-leaved trees (e.g. *Quercus crispula*, *Betula platyphylla*) and evergreen needle-leaved trees (e.g. *Abies mariesii*, *Tsuga diversifolia*). Both populations had a plant patch size of about 100 m².

### Composition of pollinator community

To determine the composition of the pollinator community associated with *L. album*, we randomly established a 2 × 2 m quadrat within each patch for each observation day. We conducted seven censuses at P1 (May 20, 31; June 7, 12, 18, 21, 22) and 11 censuses at P2 (May 29, 31; June 3, 7, 12, 15, 18, 21, 22, 30; July 7) during the flowering season of *L. album* in 2016. We conducted one census per day, taking 60 min for each census, between 07:00 and 16:00 at times of high bumblebee activity. There was no variation in bumblebee activity during this time. During each census, we counted the bumblebees flying into the quadrat, and confirmed that each bumblebee visited *L. album* flowers.

### Flowering phenology and temporal variation in corolla length in *L. album*

In each quadrat, we marked all *L. album* inflorescences and counted the flowers that had newly opened to accurately record flowering phenology. Furthermore, following the method of Hattori et al. (2015) we measured the total length of each corolla (i.e. length from the base to the tip of the corolla) with a digital calliper (precision 0.01 mm) from May 31 to June 26 in P1 and from May 20 to July 7 in P2. We randomly selected 20 inflorescences from P1 and 44 inflorescences from P2, and then 1–15 flowers from each inflorescence were measured. We observed that *L. album* was mainly visited by *Bombus consobrinus* throughout the flowering period in this study, and that the predominant caste of pollinator bumblebees tended to change from queens to workers as the flowering period progressed. To find out whether corolla length decreased as the season progressed, we used a linear mixed-effects model. In this analysis, the day number from May 16 was included as a fixed-effect factor, and the position of each flower within the inflorescence (the whorl number above the bottom whorl in the inflorescence) was included as a random effect, because flower size can vary within inflorescences due to architectural effects (Diggle 1995). This analysis was performed in R v.3.1.1 (R Development Core Team 2014).

## Effect of size-matching between flowers and pollinators on the seed set

To evaluate the effects of flower-pollinator size matching on seed set in P2 in 2016, we randomly established a 1 × 1 m quadrat within the P2 patch and bagged 15 young inflorescences that only had buds within the quadrat in fine nylon mesh bags (0.75 mm) until one-visit experiment for estimation of female flower fitness from June 12 to June 22. We then removed the bags to allow bumblebees to visit the freshly opened flowers. After each visit by a bumblebee, we caught the bumblebee, identified it to species level and measured its head length and proboscis length (*B. consobrinus* workers (n = 11), *B. diversus* queens (n = 31), *B. honshuensis* queens (n = 3), and a *B. honshuensis* worker (n = 1)). We also measured the length of the flowers that were visited by the bumblebee. Size-matching between bee and flower was calculated as follows: corolla length − head length − proboscis length. The measure indicates the position of the bee's head in relation to the anthers and stigma of the flower.

Soon after each visit by a bumblebee, we bagged the flowers again and counted the number of seeds after fruit maturation. Then, we estimated seed set by taking the number of seeds and dividing it by four, because Lamiaceae species have four ovules (Burd et al. 2009). To determine
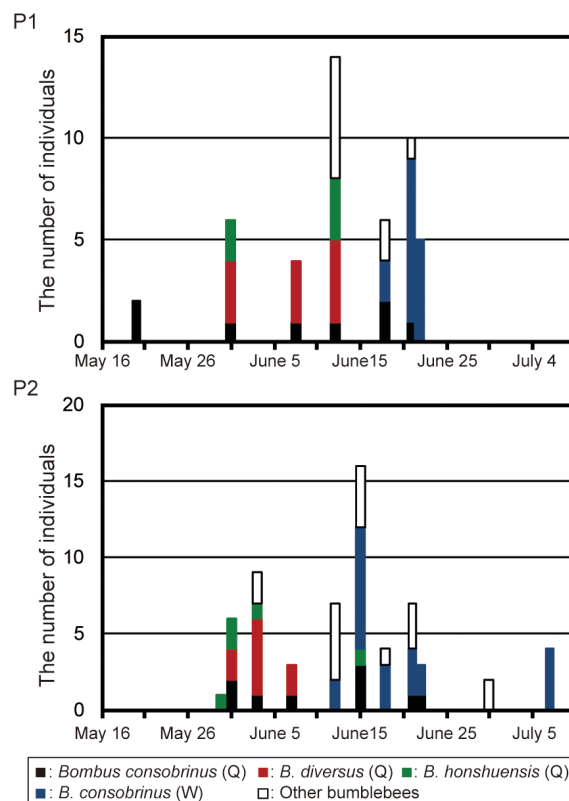


**Figure 1** – Temporal changes in the composition of the pollinator guild of *Lamium album* in the two study populations P1 and P2. The different colour bars indicate different bumblebee species and castes.

the effect of flower–pollinator size matching on seed set, we used a generalized linear mixed-effects model (GLMM) with a binomial error distribution and logit link in the R package lme4 (Bates et al. 2015). In this analysis, the flower-pollinator size match index was included as a fixed-effect factor, and the inflorescence was included as a random effect. Flowers were not always pollinated by bumblebees after removing the bag from the inflorescences. In order to evaluate the effect of autonomous self-pollination on seed set (n = 65), we also bagged non-pollinated flowers again. The seed set of non-pollinated flowers was very low (0.05), thus *L. album* flowers require visitation by pollinators for seed set.

## RESULTS

### Temporal variation in the composition of pollinator communities

We observed pollination of *L. album* by different bumblebee species and castes in two different *L. album* populations. In both populations, the primary pollinators were *B. diversus* queens, *B. honshuensis* queens, and *B. consobrinus* queens and workers (fig. 1). Especially at the peak of the flowering period (late May–mid-June; fig. 2), mainly queens visited *L. album* flowers in both populations (P1, queen visitation percentage = 91 ± 9%; P2, queen visitation percentage = 67 ± 18%). Conversely, at the end of the flowering period (mid-June–early July; fig. 2), *L. album* flowers in both populations were mainly visited by *B. consobrinus* workers (P1, queen visitation percentage = 14 ± 10%; P2, queen visitation percentage = 10 ± 7%).

### Temporal variation in the number and size of flowers

In both populations, we observed newly-opened flowers throughout the observation period. The peak of the flowering period was in early June (fig. 2). Corolla length significantly decreased as the season progressed in P1, but did not change over the flowering period in P2 (P1: $\beta$ = -0.044, t = 2.88, p = 0.005, P2: $\beta$ = -0.003, t = 0.279, p = 0.782; fig. 3). In P1, the average corolla length difference between the peak and the end of the flowering period (average corolla length ± s.e. on June 7 = 26.16 ± 0.13 (n = 117), average corolla length ± s.e. on June 21 = 24.88 ± 0.40 (n = 16)) was only about 2 mm. This difference is smaller than the size difference between the bumblebee castes (fig. 4).

### Effect of flower–pollinator size matching on seed set

In the experiment to determine the effect of the flower-pollinator size match on the seed set, 46 bumblebees (11 *B. consobrinus* workers, 31 *B. diversus* queens, 3 *B. honshuensis* queens, and a *B. honshuensis* worker) visited *L. album* flowers. The degree of size matching between flower and pollinator affected seed set ($\beta$ = -0.49, z = 2.98, AIC = 195.8, p = 0.003; fig. 5). Large-sized bumblebees (i.e.
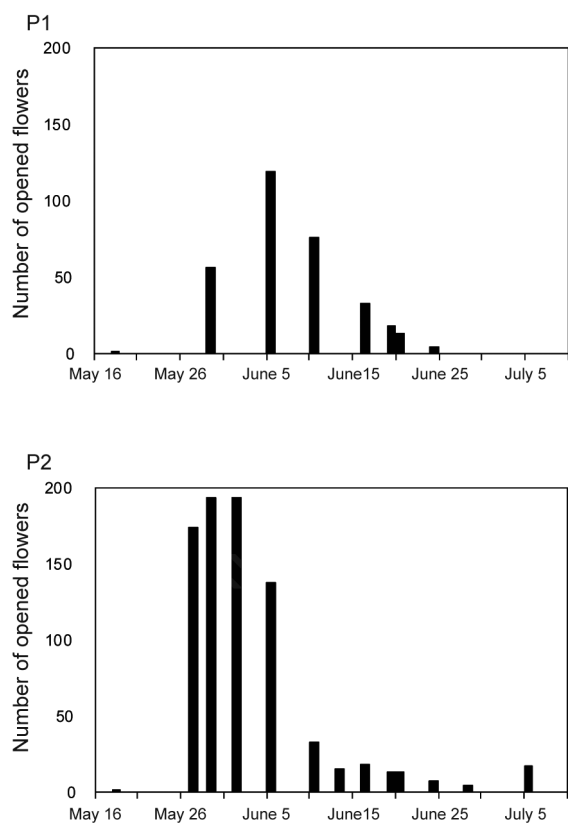


**Figure 2** – Flowering phenology of *Lamium album* in the two study populations P1 and P2. Black bars show the number of newly opened flowers.
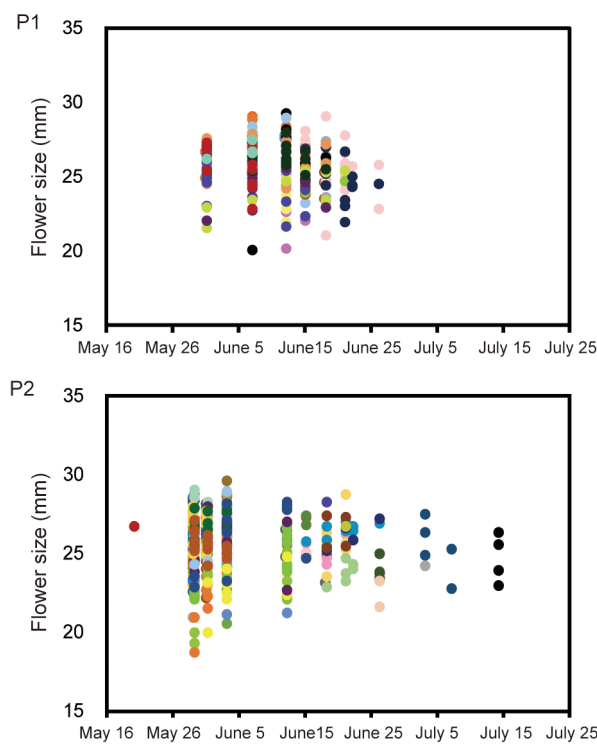


**Figure 3** – Temporal variation in corolla length (mean ± s.e.) of *Lamium album* in the two study populations P1 and P2. The different dot colours indicate different inflorescences of *L. album*.
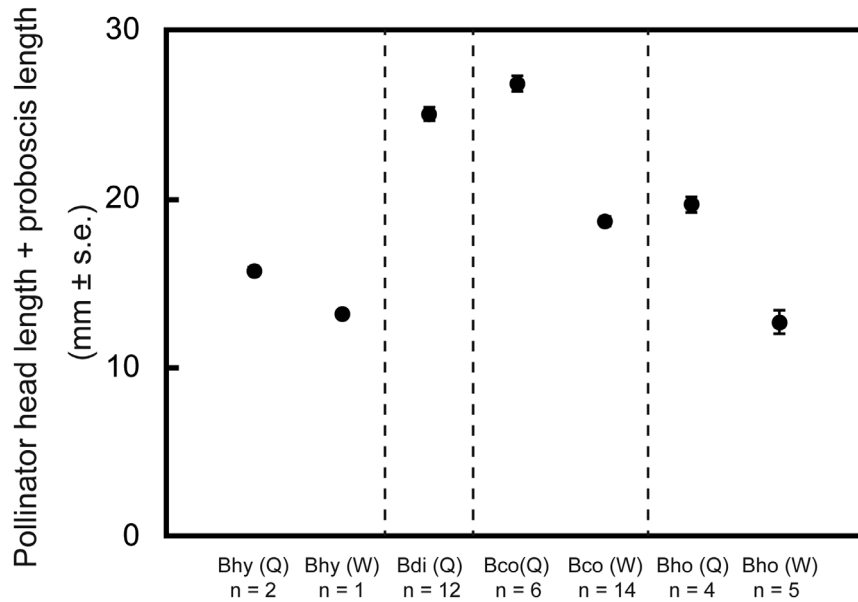
**Figure 4** – The total length of the pollinator head plus proboscis (mean ± s.e.) in P2. Bhy: *Bombus hypocrita*, Bdi: *B. diversus*, Bco: *B. consobrinus*, Bho: *B. honshuensis*, Q: Queen, W: Worker

queens) were more effective pollinators of *L. album* than small-sized bumblebees (i.e. workers) because seed set was highest when size matching was around zero.
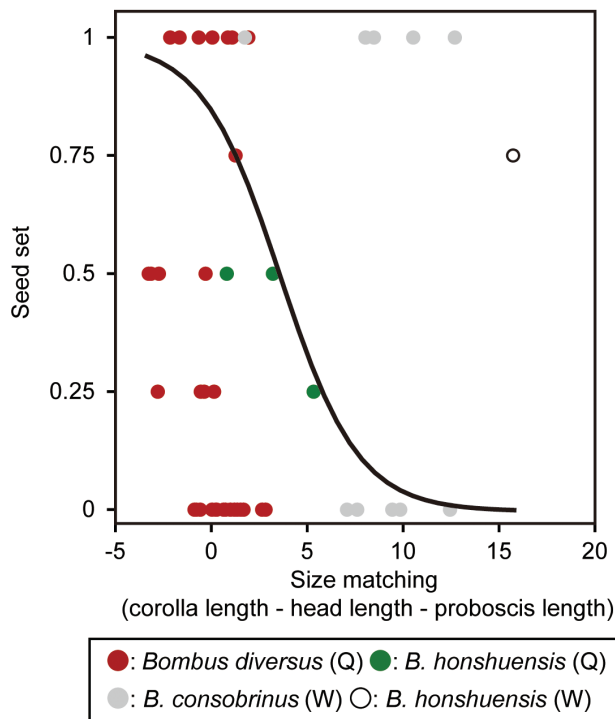


**Figure 5** – Relationship between the seed set of *Lamium album* flowers after one visit by a bumblebee and plant-pollinator size matching (corolla length – head length – proboscis length). The solid line is the predicted regression model by the generalized linear mixed-effects model.

## DISCUSSION

The adaptation of floral structures to pollinators is generally recognized as a key mechanism promoting the diversification of plants (Johnson 2006; Van der Niet et al. 2014). To confirm this mechanism, several studies have demonstrated pollinator-mediated microevolution in a variety of plant species (Nagano et al. 2014; Sun et al. 2014; Kuriya et al. 2015; Newman et al. 2015). For example, *Nerine humilis* (Jacq.) Herb. shows geographic variation of floral traits that is an adaptive response to geographic variation in the pollinator community (Newman et al. 2015). On the other hand, variation of floral traits is sometimes a response to factors that are not linked to pollination (Strauss & Whittall 2006; Hattori et al. 2016). Therefore, studies of what influences floral traits in many animal-pollinated plants are needed to fully understand the diversification of plants.

In our study, the local pollinator community of *L. album* seasonally changed from bumblebee queens to workers. This change is linked to the life cycle of bumblebees. Until the colonies attain a sufficient size, the queen has to collect and store pollen for her progeny by herself. After workers emerge, the queen stays in the nest and takes care of her progeny. Therefore, spring-flowering plants with a long flowering phenology, such as *L. album,* experience temporal variation in pollinator size. Previous research has shown that spatial variation in pollinator size can lead to local adaptation of flower size within a fine-scale landscape (Nagano et al. 2014; Sun et al. 2014; Kuriya et al. 2015).

The results showed that bigger bumblebees (queens) were more effective in pollinating *L. album* flowers than smaller bumblebees (workers). This suggests that corolla length of *L. album* would only be under selection when visited by bumblebee queens. In fact, the bumblebee queens proved

to be more effective pollinators than bumblebee workers in this study. When bumblebee queens visited the flowers, we observed that they thrusted themselves into the flower and collected nectar. At that time, the head of the bee touched the anthers, and pollen adhered to the head. In contrast, when bumblebee workers visited flowers, their heads did not touch the anthers because of the size mismatch. If "the most effective pollinator principle" (Stebbins 1970) would apply, the corolla length of *L. album* should be somewhere between bumblebee queen size and worker size, because the numbers of workers and queens visiting the *L. album* observation patch were not so different (fig. 1).

Why does the corolla length of *L. album* match only bumblebee queens and not workers? Here, we pose three hypotheses: (1) repeated flower visits by workers compensate for low pollen deposition; (2) visitation patterns of bumblebee workers have negative effects on cross-pollination in *L. album*; (3) there may be developmental limitations on how much flower size of *L. album* can differ within or among inflorescences.

The first hypothesis assumes that workers visit more flowers after entering the patch. Indeed, we observed that the flower visitation frequency of workers tends to be higher than that of queens (pers. obs.). In this study, the pollination efficacy was tested by looking at seed set after one visit only. Our result showed that the pollination efficacy per visit of bumblebee queens is higher than for workers. But, if the flowers are visited more often by workers and seed set increases with the number of visits, this would compensate for the lower pollen deposition per visit.

The second hypothesis assumes inbreeding depression (Charlesworth & Charlesworth 1987) and/or pollen discounting (Harder & Barrett 1995) through pollen deposition by workers that probe flowers in sequence on neighbouring flowers. In plants that produce many flowers such as *L. album*, pollinators that visit flowers within inflorescences cause higher rates of selfing (Kudo et al. 2011; Karron & Mitchell 2012). Furthermore, the workers tend to visit more nearby flowers than the queens (Kudo et al. 2011). Therefore, the visitation patterns of workers may promote inbreeding and reduce the amount of pollen that was exported to other individuals (i.e. pollen discounting) if workers had a higher rate of pollen deposition. A less than perfect size match with bumblebee workers could reduce these negative effects.

The third hypothesis assumes that *L. album* cannot produce variation in corolla length through developmental constraints. Flowers are complexes of multiple morphological traits that are functionally and developmentally interrelated (Wessinger & Hileman 2016). In evolutionary history, *L. album* may not have acquired any mechanisms to change the developmental program of an inflorescence so that later inflorescences would have smaller flowers.

Further study is needed to confirm the effect of various factors on corolla length in *L. album* (e.g. repeated flower visits by workers, outcrossing rates over the course of the season, developmental limitations on how much flower size can differ among inflorescences of a single genotype, etc.). Especially, the effect of genetic structure within the population on the corolla length should be tested. Genetic structure may influence corolla length in our study because *L. album* produces clonal ramets and our study was conducted in a limited area, so most of the inflorescences were likely produced by the same genet. The seeds of *L. album* are dispersed by ants (Oberrath & Böhning-Gaese 2002), and this type of seed dispersal increases genetic diversity within the population. In further studies, it would useful to also include the genetic identity of the inflorescences in the analysis of flower size.

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