COMPARISON OF FLOWER-VISITING BEHAVIOUR OF BUMBLEBEES AND SWALLOWTAIL BUTTERFLIES TO THE JAPANESE AZALEA (*RHODODENDRON JAPONICUM*)

Keigo Takahashi[1*], Takao Itino[2, 3]

[1]*Graduate School of Science and Technology,*
[2]*Department of Biology, Faculty of Science and*
[3]*Institute of Mountain Science, Shinshu University, Matsumoto, Nagano 390-8621, Japan*

Abstract—Examination of the efficacy of pollinators that are not morphologically matched to a flower's shape can deepen our understanding of the main pollinators of a plant species. In central Japan, *Rhododendron japonicum* is visited much more frequently by bumblebees than by butterflies although its flower shape is more suited for pollination by large butterflies. Here, we observed flower-visiting behaviour of *Bombus diversus* queens and the alpine black swallowtail butterfly, *Papilio maackii*, and compared their efficacy as pollinators. *Papilio maackii* always touched the stigma during a flower visit, whereas *B. diversus* queens did so during just 54% of their flower visits. As bumblebees visit neighboring flowers of a *R. japonicum* tree in sequence not like *P. maackii*, we hypothesized that they deliver self pollen to *R. japonicum* so that the fruit set would be low. However, the fruit set in a bumblebee-dominated area was 72.5%, significantly higher than that by hand-pollination with self pollen (31.4%). This suggests that *R. japonicum* can obtain sufficient fruits by *B. diversus* queens although its flower shape morphologically matches to pollination by swallowtail butterflies.

Keywords: bumblebees, flower shape, pollination, rhododendron, swallowtail butterflies

INTRODUCTION

Morphological matches of flower shape to primary pollinators have been an essential topic in evolutionary biology of pollination (Nilsson 1988; Stang et al. 2009; Newman 2013). Most plant species are visited by many kinds of animals in nature (Herrera 1987; Waser et al. 1996), including flower-visitors whose body shapes are not suited to the pollen deposition (Javorek et al. 2002; Fumero-Cabán & Meléndez-Ackerman 2007). For example, Epps et al. (2015) found that the flame azalea, *Rhododendron calendulaceum* (Michaux) Torrey, is only pollinated by morphologically matching large butterflies but not by frequent-flower visiting bees. It is, however, still unclear to what extent such morphologically mismatching pollinators contribute to plant pollination.

The angiosperm genus *Rhododendron* (Ericaceae) comprises more than 1,000 species (Chamberlain et al. 1996). Some *Rhododendron* species with small flowers (corolla diameter 1.5–3.0 cm) are pollinated by bumblebees or honeybees (Escaravage & Wagner 2004; Ono et al. 2008; Kudo et al. 2011), whereas those with large flowers (corolla diameter 5–7 cm) sometimes depend on swallowtail butterflies and birds for pollination (Epps et al. 2015; Huang et al. 2017). On the other hand, *R. ponticum* (corolla diameter up to 6 cm) is mainly pollinated by bumblebees (Mejias et al. 2002; Stout 2007), and 38.6% of visits by bumblebees led to contact with stigmas (Stout 2007). This indicates that bumblebees can deliver pollen even in *Rhododendron* species with large flowers.

Sixty-seven *Rhododendron* species have been confirmed in Japan (Yamazaki 1996). *Rhododendron japonicum* (A. Gray) Suringer (subgenus *Hymenanthes*, section *Pentanthera*) is a deciduous shrub distributed from Honshu to Kyushu, Japan (Yamazaki 1996). A single shrub of *R. japonicum* can have from 20 to 3,000 flowers. Flower lifespan is 7–9 days (K.T., personal observation). Similar to *Rhododendron* species pollinated by butterflies in Malesia (Stevens 1985), *R. japonicum* has large orange flowers (corolla diameter 69.5 mm on average; Takahashi & Itino 2017). Pollen grains of *R. japonicum* have strong viscosity, and *R. japonicum* has a close relative, *R. calendulaceum* that is pollinated by swallowtail butterflies (Goetsch et al. 2005; Epps et al. 2015). All of these characteristics suggest that *R. japonicum* may be pollinated by butterflies.

In our study area in Nagano, Japan, however, the visitation frequency of bumblebees (mainly the queens of *Bombus diversus* Smith) to *R. japonicum* is much higher than that of butterflies (mainly the swallowtail butterfly *Papilio maackii* Ménétriès; Takahashi & Itino 2017). The first aim of this study is to investigate whether the bumblebees and butterflies both touch the stigma during flower visits or not. Secondly, bumblebees tend to visit neighboring flowers on the same shrub in sequence, which may lead to self-pollination (De

Jong et al. 1993). As many *Rhododendron* species are self-incompatible or only weakly self-compatible (Ng & Corlett 2000; Kudo et al. 2011), bumblebees may not be ideal pollinators of *Rhododendron* species because they facilitate self-pollination. We previously found that queens of *B. diversus* tend to spend more than 10 min visiting a large *R. japonicum* shrub (Takahashi & Itino 2017). Thus, if *R. japonicum* is self-incompatible, then it could be viewed to improve the pollination effect of bumblebees that make self pollen deposition inevitable. Thirdly, if the visiting frequency of bumblebees to *Rhododendron* flowers is very high, then they can potentially function as pollinator.

The goal of this study was to determine whether *B. diversus* queens worked as the pollinator of *R. japonicum* in our study area. Accordingly, we conducted field observations and a breeding system experiment to answer the following three questions. (1) Are *B. diversus* queens inferior to *P. maackii* in the frequency with which they deliver *R. japonicum* pollen during a flower visit? (2) Is *R. japonicum* self-incompatible or self-compatible? (3) Can the basically self-incompatible *R. japonicum* achieve enough fruit set at sites where the sequentially flower-visiting bumblebees predominantly visit flowers?

MATERIALS AND METHODS

Study site

This study was conducted in Norikura (36°07′N, 137°37′E; 1,480 m a.s.l.), Matsumoto, Nagano, central Japan, from 31 May to 6 July 2017, a period which encompasses the whole flowering season of *R. japonicum*. At our study site in Norikura, 200–300 *R. japonicum* plants grow in patches over a distance of about 1 km along the edge of a mixed forest dominated by *Quercus crispula* Blume and *Malus sieboldii* (Regel) Rehder. We conducted field observations and a breeding system experiment at the study site.

Sequential flower-visiting and geitonogamous pollen deposition

To compare sequential flower-visiting of the same plant between the bumblebee (*B. diversus*), and the butterfly (*P. maackii*), we investigated the number of flowers each species visited in sequence. We recorded videos of *B. diversus* queens visiting *R. japonicum* flowers on the selected shrub with a digital camera (SONY, DSC-TX30) from 12:55 to 13:00 on 11 June and from 11:00 to 13:00 on 12, 14 and 17 June 2017. We began recording a video as soon as a bumblebee began visiting the plant and continued recording while the bumblebee visited flowers on that plant. We also counted the number of flowers visited by the bumblebee in sequence. When a butterfly visited a shrub, we observed its behaviour and also recorded the number of flowers on the plant that it visited in sequence (4 h per day for a total of 40 h). To compare flower visiting frequency and number of sequential visits to flowers on the same plant between *B. diversus* and *P. maackii*, we used the Wilcoxon rank sum test in R version 3.4.0 software (R core team 2017).

Insect body contacts with the anthers and stigma during flower visits

We used data from the videos described above to determine the frequency with which the body of the *B. diversus* queens (dorsal thorax, lateral thorax, ventral abdomen, or other parts) touched the stigma or anthers of a flower during flower visits (i.e., the number of flower visits during which the bumblebee touched the stigma or anthers relative to the total number of bumblebee flower visits, expressed as a percentage). Flower-visiting insects cannot access nectar unless they insert their proboscises into the nectar tube of the upper petal of *R. japonicum*. Although *P. maackii* suck nectar in correct (front) position, *B. diversus* queens suck nectar in various positions (Fig. 1). To investigate the relative body size of bumblebees in relation to flower size, we measured body length and abdomen width of *B. diversus* queens narcotized with CO_2. When *P. maackii* visited a shrub during the flower visitor observations, we also recorded whether it touched the anthers or stigma of each visited flower. We used Fisher's exact test in R version 3.4.0 software (R core team 2017) to evaluate the difference in frequency of contact with the flower stigma or anthers between *P. maackii* butterflies and *B. diversus* queens.

Breeding system experiment

To ascertain the degree of self-compatibility, we conducted an experiment comprising four hand-pollination treatments by using bagged flowers: (1) self: mature anthers from a flower were rubbed against the stigma of the same flower; (2) outcross: mature anthers from a flower were rubbed against the stigma of a flower on another plant located at least 10 m from the donor plant; (3) mixed: the entire surface of the stigma was hand-pollinated with self pollen from the same flower, and then the stigma was immediately hand-pollinated with outcross pollen from a flower on another plant at least 10 m away; (4) time-lag: half of the stigma's surface was hand-pollinated with self pollen and then 3 d later, the other half was hand-pollinated with outcross pollen. It was not difficult to hand-pollinate the two halves of the stigma separately with self pollen and outcross pollen because the *R. japonicum* stigma is large (diameter 1.56 ± 0.03 mm, mean ± S.E., $N = 40$). Treatments (3) and (4) were designed to imitate pollination conditions by bumblebees in the wild.

For this experiment, we haphazardly selected 40 *R. japonicum* shrubs along the edge of a mixed forest and bagged two different inflorescences per plant (each with 3–8 flower buds) with white nylon nets. Two flowers of the first inflorescence were used for the self and outcross treatments, and two flowers of the second inflorescence were used for the mixed and time-lag treatments. Other flowers of the inflorescences and unnecessary anthers of the target flowers were removed before the experiments.

In addition, an unbagged flower was selected from each of the 40 plants and assigned to an "open area". The 40 plants varied in the number of flowers and tree height. We estimated the visitation frequency of *B. diversus* queens in the open area flowers by a line transect census (one route, 1 km long, 4 m wide). We counted the number of queens that visited open area flowers during 09:00 to 11:00 local time on 12, 14 and 17 June. Flowers in the bumblebee-dominated area (see below) were excluded from this census.
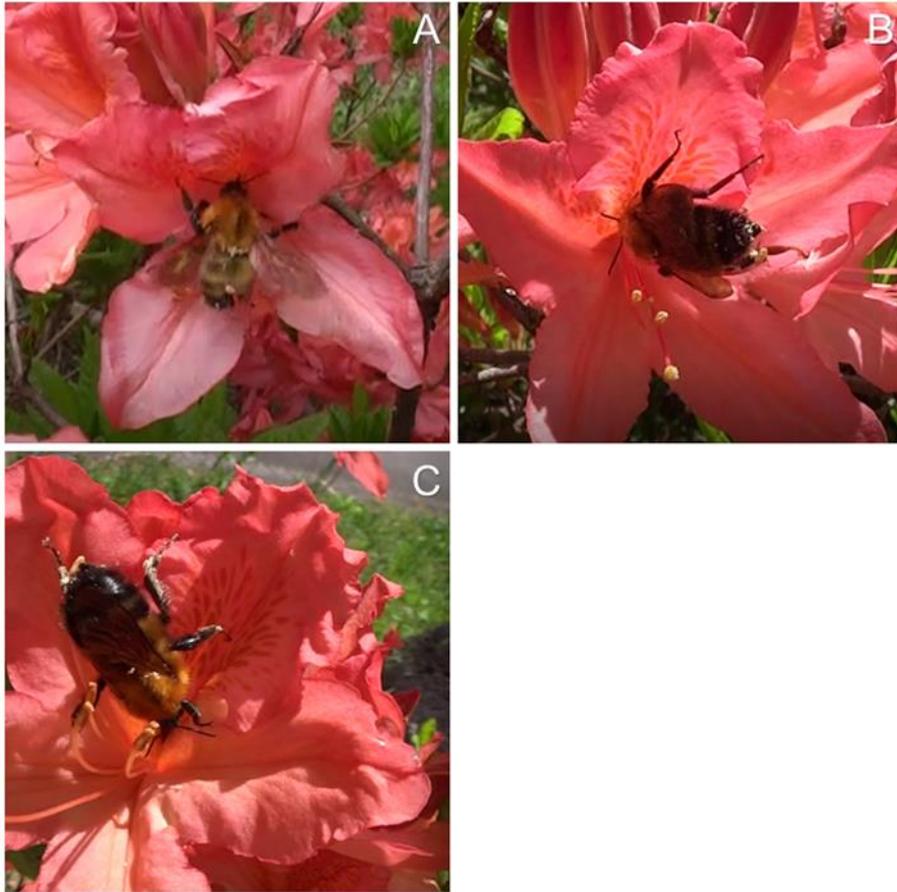
FIGURE 1. *Rhododendron japonicum* flowers and nectar-sucking postures of *Bombus diversus* queens. (A) front position. (B) side position. (C) inverted position.

We also haphazardly selected 40 flowers on four *R. japonicum* shrubs located in an area where a lot of *B. diversus* queens but no *P. maackii* butterflies were observed and assigned them to a "bumblebee-dominated area". Each of the four plants had more than 1,000 flowers during the peak flowering period. We used the data from the video recordings described above to count the number of queens that visited the bumblebee-dominated area flowers.

We checked the fruit sets of each treatment on 17 August, and compared the fruit sets between treatments and areas by using Fisher's exact test with Holm correction (R Core Team 2017).

RESULTS

Sequential flower-visiting and geitonogamous pollen deposition

On average, a *B. diversus* queen visited 17.8 ± 2.3 flowers in sequence during a visit to a single plant (mean $\pm$ S.E., the number of insects $= 30$, the number of flowers $= 534$, range 2–44 flowers), whereas a *P. maackii* individual visited 3.2 ± 1.5 flowers in sequence during a visit to a single plant (the number of insects $= 9$, the number of flowers $= 29$, range 1–15 flowers). The *B. diversus* queens visited significantly more flowers in sequence during a visit to a single plant than *P. maackii* individuals did ($W = 248$, $P = 3.2 \times 10^{-5}$).

Insect body contacts with the anthers and stigma during flower visits

During *R. japonicum* flower visits, *P. maackii* more likely touched the stigmas than *B. diversus* queens ($P = 3.2 \times 10^{-7}$; Tab. 1), but no significant difference in the frequency of touches to anthers ($P = 0.06$; Tab. 1). To suck nectar from an *R. japonicum* flower, the butterfly always faced the upper sides of the petals, so its body and/or wings rubbed against the stigma and anthers. In contrast, *B. diversus* queens (body length 22.7 ± 0.3 mm, abdomen width 9.7 ± 0.1 mm; mean $\pm$ S.E, $N = 22$) entered the flower from various directions and adopted various postures to suck the nectar; as a result, they did not always come into contact with the stigma (Fig. 1). The queens were putting together pollen in the pollen baskets on their legs, but none of them intentionally rubbed anthers with forelimbs.

Breeding system experiment

Fruit set of *R. japonicum* in the self treatment (31.4%) was significantly lower than that in the outcross treatment (88.6%; $P = 2.5 \times 10^{-5}$; Fig. 2), indicating that *R. japonicum* is weakly self-compatible in our study area. Fruit set was 54.1% in the mixed treatment and 55.6% in the time-lag treatment; in both of these treatments, fruit set was significantly lower than that in the outcross treatment (outcross vs. mixed: $P = 0.02$; outcross vs. time-lag: $P = 0.04$; Fig. 2) indicating the adverse effect of self pollen.

TABLE 1.   Percentage of *Rhododendron japonicum* flowers of which the anthers or stigma were touched by the visiting *Bombus diversus* or *Papilio maackii*. The difference in contact frequency between *P. maackii* and *B. diversus* queens was tested by Fisher's exact test: n.s., not significant; ***, $P < 1.0 \times 10^{-6}$.

|		Percentage of *R. japonicum* flowers contacted by insects (number of flower visits with contact / total number of insect flower visits)	
		Anthers	Stigma
Bombus diversus	Dorsal thorax	16.7% (52 / 311)	9.0% (28 / 311)
	Lateral thorax	24.8% (77 / 311)	5.1% (16 / 311)
	Ventral abdomen	44.7% (139 / 311)	26.7% (83 / 311)
	Other parts	32.2% (100 / 311)	19.0% (59 / 311)
	Total	87.8% (273 / 311)	54.0% (168 / 311)
Papilio maackii	Wings, ventral thorax, and abdomen	100% (26 / 26) n.s.	100% (26 / 26) ***
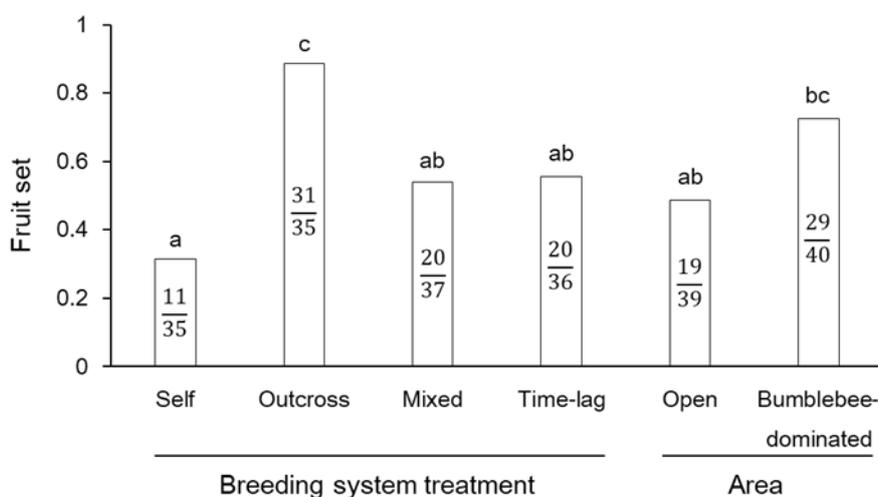
FIGURE 2.   Fruit set in the *Rhododendron japonicum*.

We observed six *B. diversus* queens visiting open area flowers during the 6-h line census, whereas during the 6-h census in the bumblebee-dominated area, we observed 30 queens visiting flowers. Fruit set in the open area was 48.7% and that in the bumblebee-dominated area was 72.5% (Fig. 2). Fruit set in the bumblebee-dominated area was significantly higher than that in the self treatment ($P = 0.007$; Fig. 2) indicating the possibility that *B. diversus* queens delivered outcross pollen to the stigmas of *R. japonicum*, at least in the area where they were abundantly observed.

DISCUSSION

Bombus diversus queens were less likely to touch the stigma during a flower visit, compared with *P. maackii* (Tab. 1). Thus, the answer of our question (1) in the introduction (Are *B. diversus* queens inferior to *P. maackii* in the frequency with which they deliver *R. japonicum* pollen during a flower visit?) was "yes". However, as the visitation frequency of

bumblebees was about ten times higher than that of swallowtail butterflies in this study site (Takahashi & Itino 2017), *B. diversus* may exceed *P. maackii* in the chance of pollen delivery. Such a visitation of bumblebees to *Rhododendron* flowers with fewer contacts with stigmas than butterflies was also reported in the interaction between *R. ponticum* with large flowers and large bumblebees (Stout 2007). On the other hand, *R. calendulaceum* with large flowers are pollinated by butterfly wings (Epps et al. 2015). Looking at the flower shape, the very long stamens and pistils of *R. calendulaceum* may account for this difference.

Regarding our question (2) in the introduction (Is *R. japonicum* self-incompatible or self-compatible?), *R. japonicum* was weakly self-compatible (Fig. 2). However, resource allocation by the maternal plant may have influenced the results of our experimental manipulation. If a maternal plant shunts more resources toward a better pollinated flower, the performance of a worse flower will be underestimated. Although *R. japonicum* could produce fruits by self pollen,

outcross pollination was important for the reproduction of *R. japonicum* (Fig. 2). *Rhododendron* species typically suffers from inbreeding depression (e.g., *R. brachycarpum*, Hirao 2010; *R. ferrugineum*, Delmas et al. 2014).

We initially hypothesized that *B. diversus* queens cannot efficiently pollinate *R. japonicum* because their geitonogamic foraging behaviour would lead to self-pollination. Indeed, *B. diversus* queens visited about six times more flowers in sequence on the same plant than *P. maackii* did. However, fruit set in the bumblebee-dominated area was similar to that in the outcross treatment (Fig. 2); it seems that bumblebees function as pollinators even though *R. japonicum* is only weakly self-compatible (Fig. 2). Because butterfly visits were not completely excluded in the bumblebee-dominated area, the answer of our question (3) (Can *R. japonicum* achieve enough fruit set at sites where bumblebees predominantly visit flowers?) was provisionally "yes". In contrast, in the mixed and time-lag treatments (which were designed to mimic pollination by self and outcross pollen-delivering bumblebee foraging in the wild), fruit set was lower than in the outcross treatment. This result suggests that fertilization by outcross pollen was partly inhibited when self pollen had already been deposited on the stigma. Why then was there the significant difference in the fruit set between the bumblebee-dominated area and the self treatment? First of all, it is possible that four plants of the bumblebee-dominated area had higher self-compatibility than 40 plants of the open area. Unfortunately, this cannot be verified by our data. Another possibility is that *B. diversus* queens may cause much cross-pollination in the early stage of their stay. Some of the *B. diversus* queens were wearing pollen of *R. japonicum* before visiting the first flower in the videos. Under this condition, cross pollen would preferentially attach to the stigma. Also, the open area included patches with few flower visiting insects, which may cause failure of pollination and consequently, the fruit set in the open area might be not so high (48.7%). Considering these results together, we infer that the frequent visits of bumblebees in the bumblebee-dominated area brought large amount of outcross pollen to the flowers and promoted the fruit set, despite the adverse effect of self pollen.

In Japan, *Rhododendron* species with small flowers such as *R. semibarbatum* Maximowicz (Ono et al. 2008) and *R. aureum* Georgi (Kudo et al. 2011) are pollinated by bumblebees. The result of this study suggests that Japanese *Rhododendron* species with large flowers can benefit from bumblebee visitation. However, in future studies, seed set of a single flower visit is required to compare pollination efficiency accurately between bumblebees and butterflies (Ne'eman et al. 2010).

As Stout (2007) stated, bumblebee species with small body size do not necessarily match flower shape of *Rhododendron*, so that stigma contacts depend on their body size. In our observation, the bumblebee workers (*B. ardens* Smith and *B. hypocrita* Pérez) collected *R. japonicum* pollen and they were too small to accomplish the pollination of *R. japonicum*. In this respect, an interesting issue for further research is the geographic corolla diameter variation of *R. japonicum* (Takahashi & Itino 2017). As bumblebee queens and swallowtail butterflies occur in spring to early summer in

Japan while workers are abundant in mid-summer, the geographic variation of flowering phenology *R. japonicum* may synchronize different-sized flower visitors and this may affect regional flower size adaptation of *Rhododendron*; it may be smaller in the region where small sized flower visitors are abundant. By investigating the factors influencing regional flower size of *Rhododendron* (e.g., pollinator size, visitation frequencies and behaviour), we will be able to shed light on the role of bees on the evolution of plant flower shapes.

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