VISITATION FREQUENCIES OF BUMBLEBEES AND SWALLOWTAIL BUTTERFLIES TO FLOWERS AND THE NECTAR SUGAR CONCENTRATION OF Rhododendron kaempferi AND R. japonicum IN MOUNTAINS OF CENTRAL JAPAN

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Abstract—Relationships between flower visitor composition and floral traits help us to understand floral evolution. Swallowtail butterflies have been assumed to be the main pollinators of Rhododendron kaempferi and R. japonicum based on their floral shapes, and R. kaempferi was reported to be pollinated by butterflies in southern Japan. In the mountains of central and northern Japan, however, bumblebees are abundant and candidate pollinators of Rhododendron. We found that visitation frequencies of bumblebees were higher than those of swallowtail butterflies to both of the Rhododendron species at three study sites in the mountains of central Japan. R. japonicum pollen adhered to the bodies of both floral visitors. Further, the nectar sugar concentration of R. kaempferi was in the preference range of bumblebees, whereas that of R. japonicum was suited to both bumblebees and butterflies. Taken together, these findings support the hypothesis that bumblebees are potentially also important pollinators of R. kaempferi and R. japonicum in mountains of central Japan, where they are more abundant than swallowtail butterflies.

Keywords: bumblebee, floral characteristics, nectar, rhododendron, swallowtail butterfly

INTRODUCTION

Relationships between flower visitor composition and floral traits can help us to understand floral evolution (Kevan & Baker 1983; Schemske & Bradshaw 1999). Suites of floral traits have evolved in response to natural selection imposed by different pollinator taxa, and these relationships are referred to as “pollination syndromes” (Johnson & Steiner 2000; Fenster et al. 2004). In recent years, the validity of the traditional pollination syndrome concept on a world scale has been challenged (Ollerton et al. 2009). The expected pollinator of some plant species on the basis of their floral traits (shape, colour, scent, size, etc.) is often not realised (Ollerton et al. 2009; Vlašínková et al. 2017). In these cases, although floral traits may have initially evolved in response to the most effective pollinator (Rosas-Guerrero et al. 2014), pollinator transitions can occur (Ollerton et al. 2009), which can result in floral traits that do not predict the current pollinator.

Rhododendron species can be pollinated by various animals and thus exhibit a diversity of floral traits and a range of pollination syndromes. Rhododendron calendulaceum (subgenus Pentanthera, section Pentanthera), which has big orange petals, a long pistil, and long stamens, is butterfly-pollinated (Epps et al. 2015). R. floccigerum (subgenus Hymenanthes, section Pontica), which has red petals with very short pistil and stamens exertion, is bumblebee- and bird-pollinated (Georganos et al. 2015). On the other hand, R. aureum (subgenus Hymenanthes, section Pontica), which has medium-sized yellow flowers with relatively short pistil and stamens (Kudo 1993; Kudo et al. 2011), R. ponticum (subgenus Hymenanthes, section Pontica), which has medium-sized purple flowers (corolla diameter 3.5 to 5 cm) (Stout et al. 2006), and R. ferrugineum (subgenus Rhododendron, section Rhododendron), which has medium-sized pink flowers with relatively short pistil and stamens (Escaravage & Wagner 2004), are all bumblebee-pollinated.

The shrubs R. kaempferi (subgenus Azaleastrum, section Tsutsus) and R. japonicum (subgenus Pentanthera, section Pentanthera) have medium to large red or red-orange flowers (corolla diameter 4 and 7 cm, respectively), and a long pistil and long stamens that stick out of the corolla. Rhododendron flowers with this colour and shape have been thought to be pollinated by large butterflies, because butterflies easily recognize reddish colours and when they alight on the corolla, their ventral abdomen is suitably positioned to brush against the long pistil and stamens (Tanaka 1993, 1997, 2001). Indeed, in the Kirishima Mountains in southern Japan, R. kaempferi is frequently visited by the swallowtail butterflies Bysa alcinous and Papilio xuthus (Yokogawa & Hotta 1995). However,
swallowtail butterflies are rare in northern and mountainous regions in Japan, whereas bumblebees, the other potential Rhododendron pollinator, are abundant. When the body sizes of bumblebees match the size of Rhododendron flowers, their ventral abdomen touches the anthers and the stigma (Mojias et al. 2002). Whether *R. kaempferi* and *R. japonicum* are pollinated by large swallowtail butterflies or by bumblebees in these central mountainous regions of Japan remains unknown.

In this paper, we pose three hypotheses. First, we hypothesize that bumblebees, not swallowtail butterflies, are main flower visitors of *R. kaempferi* and *R. japonicum* in the mountains of central Japan. Second, sugar concentration of nectar of *R. kaempferi* and *R. japonicum* is high (suited to bumblebee pollination). Third, that similar selection pressures by the same pollinators, flower size of *R. kaempferi* and *R. japonicum* does not vary between populations.

**MATERIALS AND METHODS**

*Study sites*

This study was carried out in Nagano Prefecture, central Japan, for 26 days from 29 April to 10 July 2016. This period includes the peak flowering season of both *Rhododendron kaempferi* and *R. japonicum*. We investigated *R. kaempferi* at sites in Ike (Ike, 36°23’N, 137°53’E; 633 m a.s.l.) and Norikura (Nor, 36°07’N, 137°53’E; 1,892 m a.s.l.), and *R. japonicum* at sites in Nor and Utsukushigahara (Uts, 36°14’N, 138°06’E; 1,892 m a.s.l.). In Ike, 50 *R. kaempferi* plants grew within an area of 50 m × 50 m at the edge of a mixed forest in which *Pinus densiflora* was the dominant tree species. In Nor, a few dozen *R. kaempferi* plants grew sporadically over a distance of about 1 km along the edge of a mixed forest in which *Quercus crispula* and *Malus sieboldii* were dominant tree species. Also in Nor, 200–300 *R. japonicum* plants grew sporadically along the forest edge. In Uts, 500–600 *R. japonicum* plants grew in pastureland within an area of about 200 m × 800 m. We haphazardly selected 15–20 *R. kaempferi* and *R. japonicum* plants at each study site for the following investigation.

*Visitation frequencies and pollen adhesion on flower-visiting insects*

Visitation frequencies of insects, calculated as per hour per flower, were counted for a total of 10 hours for the two plant species between 0800 and 1600 local time (LT) at each site. We regarded an insect as a flower visitor if it inserted its mouthpart into at least one flower nectar tube in a patch. The number of *R. kaempferi* flowers in a patch was 396.8 ± 985.4 (*N* = 8) in Nor and 53.4 ± 42.8 (*N* = 7) in Uts. The visitation frequencies of *Bombus* species and Papilionidae species were compared by using a paired *t*-test on log-transformed values of visitation per flower per hour. Additionally, we observed the flower-visiting behaviour of *Papilio maackii* and *Bombus diversus* at a *R. japonicum* patch with 3,200 flowers in Nor for one hour. We recorded the time spent by insect individuals at the patch and the number of flowers visited per foraging bout.

To quantify the number of *R. japonicum* pollen grains that adhered to the bodies of the visiting insects, we placed round seals (8 mm in diameter, adhesive on one side, NICHIBAN ML-151) on the ventral abdomen of *P. maackii* (*N* = 1) and on the dorsal and lateral thorax and the ventral abdomen (i.e., three seals per individual) of *B. diversus* queens (*N* = 4) in Nor on 15 June 2016. We then counted the number of pollen grains adhering to the seals under a stereoscopic microscope (Nikon SMZ1500). In *R. kaempferi*, we captured a queen and a worker of *B. ardens*, and an individual of *P. maackii* in Ike and confirmed pollen grains of *R. kaempferi* attached to their bodies with the naked eye.

Although we could not test pollination efficiency directly (e.g., by single visit experiments, or other ways of measuring pollen deposition), we collected visitation data and floral characteristics data (describe below) as proxies.

*Daytime and night-time nectar collection quantities*

To determine whether *R. kaempferi* produced more nectar in the daytime or at night, we used 0.5 µL capillary tubes (Drummond Scientific) to remove all the standing crop of nectar from 12 haphazardly selected *R. kaempferi* flowers in Nor at 16:00 LT on 31 May and then covered each flower with a nylon net to prevent insect access. The next morning, at 07:30 LT, we measured the nectar accumulated during the previous night in nine flowers (three flowers fell off the plant during the night). Then we recovered each of the nine flowers with a nylon net and, at 16:00 LT, measured the nectar volume in six flowers (three flowers fell off the plant during the day) accumulated in the daytime. We compared night-time and daytime nectar collection quantities by the Wilcoxon rank sum test (R version 3.4.0; R core team 2017).

*Sugar concentration of nectar*

We haphazardly selected 3–20 flowers (each flower from a different plant) per site per species and measured the sugar concentration of the standing crop of nectar at 08:00 LT (three flowers of *R. kaempferi* in Ike, three flowers of *R. kaempferi* in Nor, three flowers of *R. japonicum* in Nor, and 20 flowers of *R. japonicum* in Uts) by using 2µL capillary tubes (Drummond Scientific) and hand-held refractometer (Bellingham and Stanley). To investigate the pattern of the large variation of sugar concentration in *R. japonicum* in Uts, we increased the sample size there. In addition, we haphazardly selected three flowers from each of four randomly selected *R. japonicum* plants in Uts and measured the sugar concentration of the standing crop of nectar between 09:00 and 12:00 LT, and between-plant differences in nectar sugar concentration were tested by using one-way ANOVA (R version 3.4.0; R core team 2017).

*Floral size*

We haphazardly selected 20 flowers (one from each of 20 plants) per site per plant species (but 15 *R. kaempferi*...
flowers in Nor), and measured corolla diameter, lengths of the shortest and longest stamen (among five stamens), style length, and nectar tube length. Corolla diameter was defined as the distance between the top centre of the upper petal and the midpoint between the apices of the two lower petals (Fig. 1C). Nectar tube refers to the tubular structure at the base of the upper petal (Fig. 1A). Intraspecific and between-site differences in floral sizes were tested by using an unpaired two-tailed t-test.

RESULTS

Visitation frequencies and pollen adhesion on flower-visiting insects

Two species of butterfly were observed to visit Rhododendron kaempferi in Ike (Parnassius glacialis and Papilio maackii), whilst none were observed to visit R. kaempferi in Nor. P. maackii was also observed to visit R. japonicum in Nor, whilst no butterflies were observed to visit R. japonicum in Uts (Fig. 2). Three species of bumblebee were observed: R. kaempferi was visited by Bombus ardens in Ike and B. diversus in Nor; and R. japonicum was visited by B. ardens, B. hypocrita and B. diversus in Nor and B. hypocrita in Uts.

Bumblebees were more frequent visitors to R. kaempferi and R. japonicum than swallowtail butterflies at all study sites although the difference was only statistically significant for R. japonicum in Nor (Fig. 2).

Our limited observations of 3,200 flowers over 1 hour suggested that bumblebees stayed longer in a flower patch and visited more flowers per hour than butterflies. One individual of P. maackii perched and took nectar from only two flowers within a minute whilst three B. diversus queens stayed for 12, 15, and 18 minutes, visiting about 10 flowers per minute.

Queens of B. diversus had 44.5 ± 35.5 (mean ± S.E., N = 4) pollen grains per seal on the dorsal thorax, 160.5 ± 67.3 on the lateral thorax, and 125.5 ± 47.8 on the ventral abdomen. The swallowtail P. maackii had 857 (N = 1) pollen grains per seal on its ventral abdomen (Fig. 3).

Nectar collection quantities during the day and night

In Nor, a total of 0.005 ± 0.004 μL (mean ± S.E., N = 9) of R. kaempferi nectar was extracted after flowers had been bagged overnight (for 15 and a half hours), and a total of 0.289 ± 0.049 μL (N = 6) at the end of the following day (8 and a half hours, W = 0, P < 0.001).

Sugar concentration of nectar

The sugar concentration of R. kaempferi nectar was 54.7 ± 4.9% (mean ± S.E., N = 3) in Ike and 51.3 ± 0.3% (N = 3) in Nor, whereas that of R. japonicum nectar was 45.7 ± 0.7% (N = 3) in Nor and 30.2 ± 1.9% (N = 20) in Uts. In Uts there was a significant difference in nectar

![Flowers of Rhododendron kaempferi (A & B) and R. japonicum (C & D): (A) Front and (B) side view of a flower of R. kaempferi. In (A), there is a reddish nectar guide and a nectar tube in the position indicated by the white line. In (B), the pistil and five stamens can be seen to stick out of the corolla and curve upward. (C) Front and (D) side view of a flower of R. japonicum. In (C), the solid white line shows how we measured corolla diameter. The dashed line connects the tips of the two lower petals.](image-url)
concentration between the four plants tested (mean ± S.E. in Plant A: 23.3 ± 4.1%, B: 17.0 ± 3.5%, C: 22.0 ± 1.0%, and D: 33.7 ± 1.2%; d.f. = 3, F = 6.27, P < 0.05).

**Floral size**

Regional differences in floral size were not detected in *R. kaempferi* (corolla diameter: t = 0.69, P = 0.49; minimum stamen length: t = 0.32, P = 0.75; maximum stamen length: t = 0.46, P = 0.65; style length: t = 1.10, P = 0.28; nectar tube length: t = 1.26, P = 0.22; Tab. 1), but detected in *R. japonicum* flowers were larger in Nor than in Uts in terms of corolla diameter (t = 2.78, P < 0.01), maximum stamen length (t = 2.46, P < 0.05), and style length (t = 4.38, P < 0.0001; Tab. 1).

**DISCUSSION**

Bumblebees were more frequent visitors than swallowtail butterflies to *Rhododendron kaempferi* and *R. japonicum* spp., although the difference is only significant in one of four cases. Yokogawa and Hotta (1995) reported that the most frequent flower visitor to *R. kaempferi* was the swallowtail butterfly *Byasa alcinos* in the Kirishima Mountains, Kagoshima Prefecture (31°53'N), southern Japan. The species diversity and abundance of swallowtail butterflies are higher in southern Japan (Kiritani 2006), whereas those of bumblebees are higher in northern and mountainous central Japan (Goulson 2003). Therefore, the main pollinator of *R. kaempferi* may change between mountainous central Japan and southern Japan (over a distance of 777 km) according to local bee and butterfly diversity and abundance.

Although Tanaka (1993, 1997, 2001) inferred that floral shape of *Rhododendron* spp. is adapted to swallowtail butterfly pollination in Japan, pollen grains also adhere to the bodies of the flower-visiting bumblebees, suggesting that they could also be pollinators of *R. kaempferi* and *R. japonicum*. When bumblebees hovered in front of the corolla, they changed their position frequently and pollen grains adhered all parts of their bodies. On the other hand, when the swallowtail butterfly *P. maackii* visited a flower, it landed on the pistil and stamens and sucked nectar with its legs and the ventral side of abdomen being in contact with the stigma and anthers, which extend outward horizontally with slightly curving tips in both *R. kaempferi* and *R. japonicum*. This more precise pollen placement may have implications for the relative efficiency of butterflies vs. bumblebees as pollinators, but this requires further investigation.

The adaptation of nectar production time has been reported to be a component of a pollination syndrome (Cruden & Hermann-Parker 1979; Inoue et al. 1991). *R. kaempferi* nectar was mainly produced in the daytime, making it suited to daytime pollinators such as swallowtail...
butterflies and bumblebees, although it would have been better to add a treatment reversing the order between daytime collection and night-time collection to test production in day vs. night independently. We do not know whether the pollinator-related traits of *R. kaempferi* and *R. japonicum* changes between populations depending on the abundance of and adaptation to the effective pollinator group.

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