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BRIEF REPORT

The relative importance of diurnal and nocturnal pollinators of *Platanthera hologlottis* Maxim. (Orchidaceae)

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ABSTRACT

Most flowering plants are visited by various pollinator insects. To understand floral specialization for pollinators, the relative importance of different flower visitors to the focal plant species should be revealed. In the present study, we observed the insects that visited the orchid *Platanthera hologlottis* throughout the day and night using interval timer photography to reveal the relative importance of diurnal and nocturnal flower visitors. We observed visitation by both diurnal (e.g. the butterfly *Ochlodes ochraceus*) and nocturnal (e.g. the moth *Thysanoplusia intermixta*) insects and examined their relative contribution to fruit production and pollinarium removal experimentally. Results showed that the fitness was higher in flowers visited by nocturnal insects than in those visited by diurnal insects. These results suggest that the floral traits of *P. hologlottis* may be specialized for nocturnal flower visitors rather than diurnal flower visitors.

Introduction

Most flowering plants depend on animals, mostly insects, for their pollination (Ollerton et al. 2011). The relationship between plants and pollinator insects (i.e. pollination mutualism) strongly influences their reproductive success. To improve reproductive success, floral traits can become ecologically, evolutionary, and phenotypically specialized for their pollinators (Boberg and Ågren 2009; Newman et al. 2015; Armbruster 2017). For example, the evolution of long nectar spurs in *Aquilegia* was driven by a pollinator shift associated with changes in tongue length of pollinators (Whittall and Hodges 2007). Additionally, Sletvold et al. (2012) reported that diurnal pollinators mediated stronger selection on traits influencing floral display than nocturnal pollinators and that this selection varied between populations of *Gymnadenia conopsea*. These studies also showed that different pollinator species exerted different selection pressures on floral traits. Therefore, to understand floral specialization, it is important to reveal the pollinator species that exert selection pressure on the plant species under investigation.

In some situations, flowers are visited by different pollinator insects, and the specific species of pollinators change not only among populations (Nagano et al. 2014; Kuriya et al. 2015; Hattori et al. 2016) but also within a population (Wolff et al. 2003; Cordeiro et al. 2016; Funamoto and Ohashi 2017). For example, *Isertia laevis* (Rubiaceae) is pollinated mainly by hummingbirds (Trochilidae) during the day and by hawk moths at night. Furthermore, the reproductive success per visit of *I. laevis* pollinated by hawk moths was significantly higher than that of those pollinated by hummingbirds (Wolff et al. 2003). Funamoto and Ohashi (2017) reported an adaptation to nocturnal moths in *Adenophora triphylla* var. *japonica* (Campanulaceae) that have flowers that appear to

fit with a bee-pollination syndrome but are visited by diurnal and nocturnal insects. Although several studies have provided support for pollination syndromes (Danieli-Silva et al. 2012; Murúa and Espíndola 2015; Strelin et al. 2016), the pollination syndrome does not predict the pollinators of most plant species (Ollerton et al. 2009). Furthermore, secondary pollinators are common and play important roles in plant reproduction (Rosas-Guerrero et al. 2014). Therefore, to understand floral specialization for pollinators, it is necessary to observe pollinator species throughout the day and night to identify the relative importance of pollinator species to a focal plant.

It is sometimes difficult to directly observe nocturnal pollinators because their flower visitation frequency is very low (Suetsugu and Fukushima 2014). To overcome this problem, there has been a recent increase in studies using interval photography to observe nocturnal pollinators (e.g. Suetsugu and Tanaka 2013; Suetsugu et al. 2015). In the present study, we observed flower visitors to the orchid *Platanthera hologlottis* (Orchidaceae) throughout the day and night using interval photography. *P. hologlottis* is a perennial herb broadly distributed in Japan (Hayashi 2009). It has upright white flowers with spurs that open from June to July (Figure 1). They have a small ridge on the midrib in front of a ca. 15-mm-long spur that partially obstructs the center of the flower (Inoue 1983; Figure 1). Furthermore, *P. hologlottis* is called 'JYAKOU CHIDORI (JYAKOU means musk in Japanese)' in Japan because their flowers are fragrant. Although it has been previously reported that *P. hologlottis* flowers were visited by more diurnal insects than nocturnal insects, the flower morphology of *P. hologlottis* is similar to that of *P. flava* and *P. ussuriensis* that are pollinated by small moths (Inoue 1983). These floral characters match a moth-

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Figure 1. (a) *Platanthera hologlottis* in the wild, (b) an enlarged photograph of *P. hologlottis* flower, (c) a visiting species of *Ochlodes ohraceus* in the day, (d) a visiting species of Plussiinae in the night.

pollination syndrome (Fægri and van der Piji 1971; Fenster et al. 2004). Thus, flower morphology may imply that *P. hologlottis* flowers are ecologically specialized for moth pollination. However, it has been previously reported that pollen vectors of *P. hologlottis* were by butterflies (*Ochlodes venata*, *Colias erate*, *Pieris melete*) mainly (Inoue 1983). It is therefore unclear whether the effective pollinators of *P. hologlottis* are diurnal flower visitors. In the present study, we hypothesized that nocturnal visitors contribute more to pollen removal and fruit production than diurnal visitors.

In such species, using interval photography to observe diurnal and nocturnal flower visitors is useful for identifying the flower visitors (Suetsugu and Tanaka 2013; Suetsugu et al. 2015). Furthermore, the experimental approach to reveal the relative contribution of diurnal and nocturnal flower visitors to fruit production and pollinarium removal is useful for gaining knowledge to identify the effective pollinators because the pollination effectiveness of flower visitors can be estimated by measuring paternal (pollen removal) and maternal (producing seeds) perspectives (Schupp et al. 2017). Thus, in the present study, we determined the relative importance of diurnal and nocturnal pollinators of *P. hologlottis*.

Material & methods

Study site

We studied a population of *P. hologlottis* in Kaida Highland, central Japan (1155 m a.s.l., 35°56'50.5"N 137°38'21.4"E). In Kaida Highland, the mean monthly temperature is 7.4 °C, and the annual amount of precipitation is 2080mm. July is the rainiest month (342 mm). Our study site contained vegetation of the montane zone (<1600 m). This vegetation comprises a mosaic forest of old-growth coniferous and old-growth deciduous broad-leaved wood (Nakashizuka et al. 1993). Additionally, we also found planted coniferous forests.

The place where we studied *P. hologlottis* was a wetland and was populated by many plant species, including *Platy*codon grandiflous (Campanulaceae) and *Myosotis scorpioides* (Boraginaceae). Here, we found about 50 individuals of *P. hologlottis* within 20 m × 20 m area. In summer 2015 and 2016, we studied the pollinator assemblage associated with the flowers of *P. hologlottis*. In summer 2016, we measured the effect of pollination during the day and night on the fitness of *P. hologlottis*.

Pollinator assemblage

To investigate the visitors to *P. hologlottis* flowers, we used three digital cameras (Optio WG-40, 30, Pentax Japan) that were set-up using the interval-programming function to automatically take pictures at 30-second or 20-second intervals. The diurnal observations began at 7:00 am and ended at 5:00 pm and the nocturnal observations began at 6:00 pm and ended at 3:00 am because sunrise was about 7:00 am and sunset at 6:00 pm in observation place. We obtained 6973 photos from 19 *P. hologlottis* plants (n = 13 in 2015, n = 6 in 2016) during the day and 6525 photos from 20 *P. hologlottis* plants (n = 13 in 2015, n = 7 in 2016) during the night in 2 observation days in each year. For observations, we focused on the same individuals of *P. hologlottis*.

Effect of diurnal/nocturnal pollinators on plant fitness

To compare the relative contribution to plant fitness between diurnal and nocturnal flower visitors, we estimated their contribution to male fitness by determining the rate of pollinarium removal per individual (number of removed pollinariu/ number of flowers), and their contribution to female fitness by determining the rate of fruit set per individual (number of fruits/number of flowers). To manipulate flower visitation from pollinators to P. *hologlottis*, we set three treatments using fine nylon mesh bags (0.75 mm): Control (D + N): open-pollinated control that pollinators could visit *P. hologlottis* flowers freely (male fitness, n = 9; female fitness, n = 10), Treatment 1 (D): pollinators could visit *P. hologlottis* flowers only during the daytime (6:00 am to

5:00 pm) (male fitness, n = 8; female fitness, n = 10), and Treatment 2 (N): pollinators could visit *P. hologlottis* flowers only during the night (6:00 pm to 5:00 am) (male fitness, n = 10; female fitness, n = 11). These experiments were conducted over 3 days. 1 month after the experiments, we observed all individuals and checked whether flowers formed a fruit or not. To measure the effect of pollinator removal of the bags, we set the bags at 9 individuals, 162 flowers (T0). By this manipulation, every flower did not produce any fruits or seeds. Thus, the bags can prevent any visitations from pollinators of *P. hologlottis* completely.

Data analysis

For the comparison of the rate of fruit set per individual and the pollinarium removal rate per individual between the control and treatment groups, the Kruskal–Wallis with Wilcoxon post-hoc test was used. All statistical analyses were conducted in JMP v. 14.0.0.

Results

In the observation site, *P. hologlottis* flowers opened from June to August. We directly observed that many lepidopteran species (Lycaenidae butterflies, Pieridae butterflies, Sphinigidae moths etc.) visited the flowers in the daytime. At night, the flowers were visited by mainly Plusiinae species.

During the day, we observed visitations by 36 insect individuals, including 24 butterflies (including *Ochlodes ochraceus* (1/24) and unknown species in Pieridae (9/24), Lycaenidae (7/24), and Hesperiidae (7/24)), and 12 moths (*Macroglossum bombylans* (1/12), *Thysanoplusia intermixta* (8/12), unknown species in Plusiinae (1/12) and other moths (2/12)) (Table 1). However, at night, we observed visitations 44 times by only moths (including unknown species in Plusiinae (13/44) (Figure 1C), *Thysanoplusia intermixta* (7/44) and other moths (24/44)) visited the flowers (Table 1). When the butterfly species visited the flowers, they held the flower. Therefore, they posed only their mouth part into the flower and sucked the nectar while on the flower. Conversely, when the moth species visited the flowers, they could not hold the flower. Therefore, they posed their head into the flower and sucked the nectar while flying (Figure 1c & d).

In the experiment, the rate of fruit set per individual significantly differed among treatments (Kruskal–Wallis test, $\chi^2 = 18.04$, $P < 0.001$; Figure 2). Although the rate of fruit set per individual did not differ between the control (D + N) and N (Wilcoxon test, $Z = 1.44$, $P = 0.15$), the rate of fruit set per individual that was pollinated by only nocturnal pollinators (N) was significantly higher than the rate per individual that was pollinated by only diurnal pollinators (D)

(Wilcoxon test, $Z = 3.84$, $P < 0.001$; Figure 2). In terms of female fitness, the contribution of nocturnal pollinators was at least twice that of diurnal pollinators.

In the experiment, the pollinarium removal rate per individual differed significantly among treatments (Kruskal–Wallis test, $\chi^2 = 13.50$, $P = 0.001$; Figure 2). Although the pollinarium removal rate per individual did not differ between the control (D + N) and N (Wilcoxon test, $Z = 0.57$, $P = 0.74$), the pollinarium removal rate per individual that was pollinated by only nocturnal pollinators (N) was significantly higher than the rate per individual that was pollinated by only diurnal pollinators (D) (Wilcoxon test, $Z = 2.92$, $P = 0.003$; Figure 2). In terms of male fitness, the contribution of nocturnal pollinators was at least 5-fold higher than that of diurnal pollinators.

Discussion and conclusion

In the present study, we revealed the insect visitors to flowers of *P. hologlottis* using interval photography. *P. hologlottis* flowers were pollinated by different lepidopteran species. Visitation by *Thysanoplusia intermixta*, which was considered to be a principle pollinator of *P. hologlottis* by Inoue (1983), was observed at all times during the day and night. However, our results indicated that *T. intermixta* does not pollinate *P. hologlottis* because only nocturnal flower visitors can influence the fitness of *P. hologlottis*. This result was caused by the lesser mechanical fit between the spur length of *P. hologlottis* and the proboscis length of *T. intermixta*. In our study site, the spur length of *P. hologlottis* was longer than the proboscis length of *T. intermixta* (Tamada et al., unpublished data). This implies that the pollinaria of *P. hologlottis* may not attach to the body parts of *T. intermixta* when *T. intermixta* visited the flower of *P. hologlottis*. Unfortunately, because we could not identify all flower visitors to species level, a principle pollinator species of *P. hologlottis* was not revealed in our study. However, our findings indicate that nocturnal pollinators, mostly moths other than *T. intermixta*, are relatively more important for *P. hologlottis*. This is no wonder because it has been previously reported that *T. intermixa* was not effective pollinator in also other *Platanthera* species (*P. japonica*) even though *T. intermixta* was higher than other flower visitors (Suetsugu and Tanaka 2013).

The misleadingly reported finding that *T. intermixta* is the main pollinator of *P. hologlottis* resulted from the previous study only focusing on the frequency of flower-visiting (quantity of pollination) and not on the effectiveness of pollination (combination of male and female fitness) (Inoue 1983). The effectiveness of pollinators should be evaluated from the various aspects because the effectiveness of pollinators can be affected by males, females, or both combined (Young 2002; Wolff et al. 2003; Schupp et al. 2017). In particular, the assumption that focusing on selection by a single 'most effective pollinator' (the most common functional group of visitors) (Stebbins 1970) sometimes leads to erroneous conclusions regarding the mechanisms of floral adaptation (Ollerton et al. 2009). Young (2002) compared the effectiveness (seed production) of diunal and nocturnal pollinators by similar experimental evaluation of our study and showed that flowers exposed only to nocturnal-visiting insects (mostly sphingid and noctuid moths) produced significantly more seeds than flowers exposed only to the diurnal-visiting insects (bees, wasps, and flies) in

Table 1 The number of visitations to *Platanthera hologlottis* by different insect taxa during both day and night.

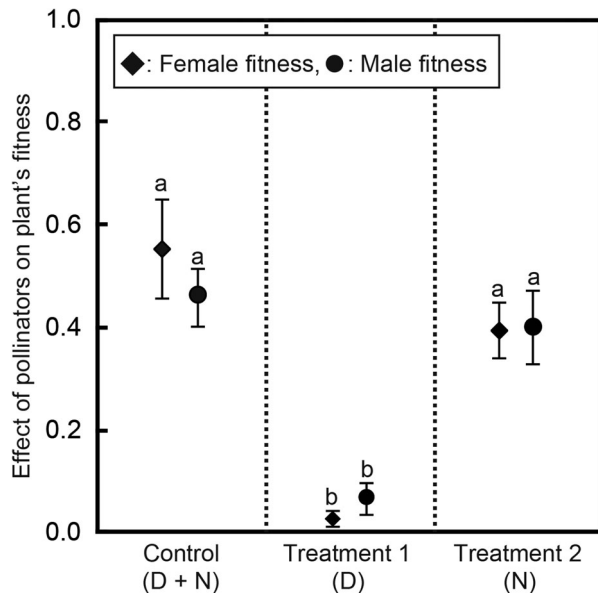| Species	Diurnal	Nocturnal
Pieridae	9	0
Lycaenidae	7	0
Hesperiidae	7	0
Ochlodes ochraceus	1	0
Macroglossum bombylans	1	0
Thysanoplusia intermixta	8	7
Plusiinae	1	13
Other moths	2	24

Figure 2. Effect of pollinators on plant fitness during both the day (D) and night (N), those with different letters are significantly different at $P < 0.05$ (Wilcoxon post hoc tests).

Silene latifolia although diurnal-visiting insects were more abundant than nocturnal-visiting insects. In our study, too, the highly frequent visiting moth (i.e. *T. intermixta*) did not contribute to produce fruits of *P. hologlottis* in comparisons with the other nocturnal-visiting insects. Therefore, focusing only on the most common pollinator does not accurately predict the agents of floral adaptation (i.e. pollination syndrome).

Our results showed that moths other than *T. intermixta* are more important pollinators than butterflies for *P. hologlottis*. This difference may result from different flower visiting behaviors between butterflies and the moths. When the butterflies visited the flowers, the pollinarium may not attach to their head because they did not pose their head into the flowers. On the other hand, when the moths visited the flowers, the pollinarium may not have got attached to the base of their head because they posed their head into the flowers. These results imply that the floral traits of *P. hologlottis* (e.g. upright white flowers and sweet fragrance) may be ecologically specialized for moths (i.e. moth-pollination syndrome) because the pollination syndrome suggests that moths favor upright white flowers with fragrance (Fulton and Hodges 1999; Hodges et al. 2003; Fenster et al. 2004). Some orchid-moth-pollinated species exhibit the same floral traits (van der Niet et al. 2011). Furthermore, fragrance traits were recognized as a kind of moth-pollinated syndrome traits. Most hawk-moth-pollinated plants show convergent evolution of emitting a sweet-smelling scent (Thompson 1994). For example, hawk-moth-pollinated tobacco plants were reported to emit greater amounts of specific fragrances during the night (Raguso et al. 2003). *P. hologlottis* may have other traits of the pollination syndrome specialized for moths other than *T. intermixta*. Further research is required to focus not only on some floral traits (e.g. fragrance, morphology, anthesis period) but also on pollinator traits (e.g. behavior, the compatibility of flower and pollinator morphology), and especially reveal whether fragrance of *P. hologlottis* flowers is favored by moths.

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