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Relative importance of diurnal and nocturnal pollinators for reproduction in the early spring flowering shrub *Stachyurus praecox* (Stachyuraceae)

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Abstract

Generalized pollination systems may be favored in early spring flowering plants, as during this period pollinator activity is unpredictable. Many previous studies have concentrated on the importance of diurnal visitors in pollination, and consequently, information on the contribution of nocturnal visitors to pollination in early spring is limited. This study was conducted to evaluate the relative importance of diurnal and nocturnal pollinators in the early spring flowering dioecious shrub *Stachyurus praecox* (Stachyuraceae), in two temperate forests in central Japan. Visitors to the female and male flowers were observed during day and night, and their relative contributions to seed set were compared. The pollinator observations revealed that the diurnal and nocturnal insects visited both male and female flowers, and that the main flower visitors were diurnal small bees and flies as well as nocturnal settling moths. The diurnal and nocturnal flower visitors also acted as pollinators, as the pollen grains of *S. praecox* were attached to the insects collected from the female flowers. Pollination experiments demonstrated that the contributions of diurnal pollinators to the seed set were higher than those of the nocturnal pollinators. The results of this study indicate that *S. praecox* has a generalized pollination system, comprising both diurnal insects and nocturnal settling moths. Although the roles of diurnal insects are more important in the pollination of *S. praecox*, nocturnal settling moths may have a complementary role in early spring.

KEYWORDS

Andrenidae, functional dioecy, gynodioecy, moth pollination, Noctuidae

1 | INTRODUCTION

Plants pollinated by multiple groups of animals might have advantages because different groups of pollinators can be complementary to each other (Blüthgen &

Klein, 2011; Macgregor, Pocock, Fox, & Evans, 2019). Generalized pollination systems are widespread in angiosperms (Funamoto, 2019; Leal et al., 2020; Waser, Chittka, Price, Williams, & Ollerton, 1996). These pollination systems are frequently found in environments

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such as oceanic islands (Kaiser-Bunbury, Traveset, & Hansen, 2010; Weissmann & Schaefer, 2017) and high mountains (Freitas & Sazima, 2006; Peng, Zhang, Xu, Li, & Sun, 2012), where pollinator availability is unpredictable. In addition, generalized pollination systems may be favored in early spring flowering plants (Alonso, 2004; Rosati et al., 2019). Pollination success in early spring could be limited by several factors (Kudo, 1995; Kudo & Cooper, 2019). Firstly, the weather in early spring is variable, and thus pollinator activity is unpredictable (Motten, 1986). Secondly, low air temperatures in early spring can strongly restrict pollinator activity (Herrera, 1995; Kudo, 1995). Furthermore, warm springs can cause phenological mismatch between pollinator emergence and flowering (Kudo & Cooper, 2019). Many studies have shown that early spring flowering plants are pollinated by a taxonomically diverse group of diurnal insects, such as bumblebees, small solitary bees, bee flies and hoverflies (Dupont & Kato, 1999; Motten, 1986; Sugiura, 2012; Wong Sato & Kato, 2017). However, relatively few studies have investigated the relative contribution of different types of pollinators to pollination in early spring flowering plant species (Huang, Song, & Huang, 2017).

Nocturnal moths, as well as diurnal insects, are known to visit several early spring flowers, such as *Daphne laureola* (Thymelaeaceae), *Narcissus* spp. (Amaryllidaceae), *Pieris japonica* (Ericaceae), *Salix caprea* (Salicaceae) and *Stachyurus praecox* (Stachyuraceae) (Abe, 2007; Alonso, 2004; Ikenoue, 1990; Ikenoue & Kanai, 2010; Jürgens, Glück, Aas, & Dötterl, 2014; Osada, Sugiura, Kawamura, Cho, & Takeda, 2003; Pérez-Barrales, Vargas, & Arroyo, 2006). Plants often achieve maximum pollination success when pollinated by both diurnal and nocturnal pollinators (Amorim, Galetto, & Sazima, 2013; Macgregor et al., 2019). Therefore, nocturnal moths may play an important role in the pollination of early spring flowers. However, the contribution of nocturnal moths to pollination has been experimentally examined in only two species of early spring flowering plants, *D. laureola* and *S. caprea* (Alonso, 2004; Jürgens et al., 2014). These studies have shown that nocturnal moths do not contribute to seed production in *D. laureola* and *S. caprea* (Alonso, 2004; Jürgens et al., 2014), as *S. caprea* was found to be pollinated by bees and the wind (Jürgens et al., 2014), and *D. laureola* by nitidulid beetles (Alonso, 2004). Thus, the roles of nocturnal settling moths in the pollination of early spring flowering plants remain poorly understood.

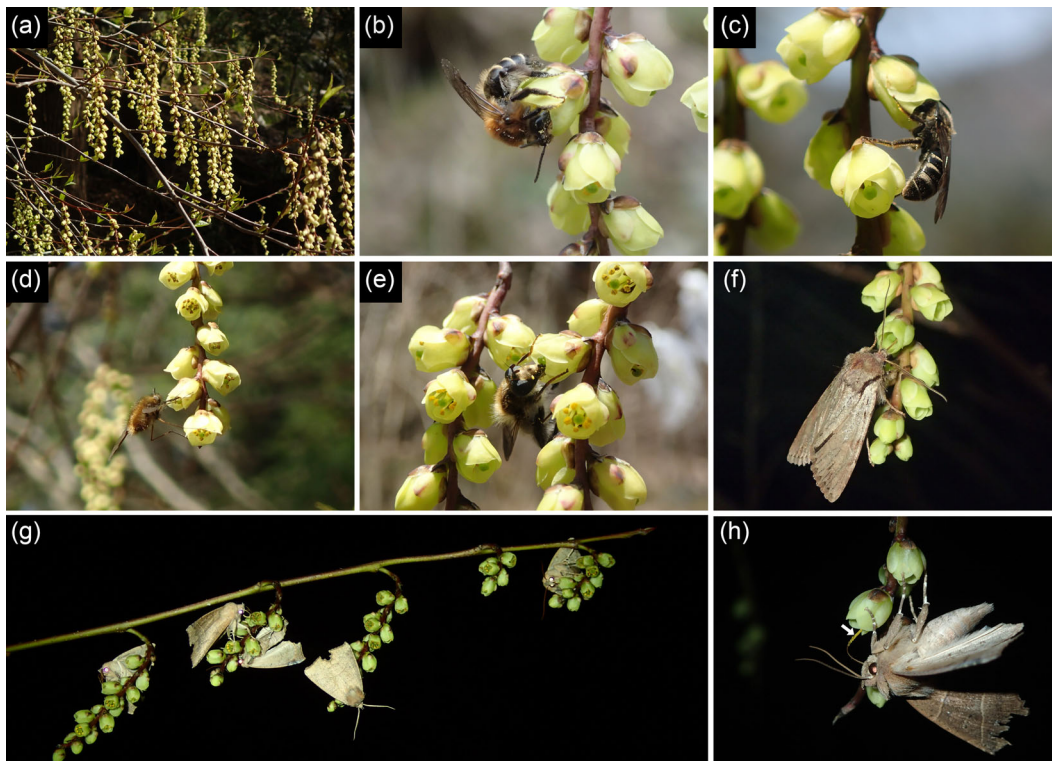


FIGURE 1 *Stachyurus praecox* flowers and flower visitors: (a) a male plant in bloom; (b) an andrenid bee visiting male flowers with pollen grains attached to the face, legs and thorax; (c) a small bee visiting female flowers; (d) *Bombylius major* visiting male flowers; (e) a hoverfly, *Criorhina* sp., visiting male flowers with clumps of pollen grains attached to its face and legs; (f) *Egira saxea* visiting female flowers; (g) *Goniocraspidum pryeri* visiting male inflorescences; (h) *G. pryeri* visiting male flowers, pollen on the proboscis is indicated by the white arrow. (a) to (f) were taken at Ashiu, and (g) and (h) were taken at Takedao

This investigation was conducted to evaluate the relative roles of the diurnal and nocturnal pollinators of *Stachyurus praecox*, a shrub that flowers in early spring (Figure 1a). The flowers of *S. praecox* attract diverse insects, including small bees, hoverflies and bee flies during the day (Abe, 2007), whereas settling moths visit the flowers at night (Ikenoue, 1990; Ikenoue & Kanai, 2010). Thus, both diurnal and nocturnal pollinators may be involved in the pollination of flowers. However, the relative contributions of diurnal and nocturnal pollinators to the seed production of *S. praecox* have not previously been investigated. This study aims to address the following question. (a) Do both diurnal insects and nocturnal moths visit flowers across years and sites? (b) Are both diurnal and nocturnal flower visitors carrying pollen grains of *S. praecox*? (c) Do both diurnal and nocturnal flower visitors contribute to the seed production of *S. praecox*?

2 | MATERIALS AND METHODS

2.1 | Species and sites

Stachyurus praecox is a deciduous shrub species that is endemic to Japan and is commonly found at the edges of its temperate forests (Takahashi, 2000). Although *S. praecox* has both female and morphologically hermaphrodite flowers, it is functionally dioecious, as the hermaphrodite flowers only produce a few fruits (Abe, 2007). We therefore define the morphologically hermaphrodite flowers as male flowers. Both female and male flowers produce nectar. The number of flowers per female and male inflorescence was 14.8 ± 6.1 (mean $\pm$ standard deviation [SD]; range, 2–28; 88 inflorescences from two individuals) and 13.6 ± 9.0 (range, 2–36; 77 inflorescences from two individuals), respectively.

The fieldwork for this investigation was conducted in two temperate forests: Takedao, Hyogo ($34^{\circ}51'N$, $135^{\circ}18'E$; 100 m above sea level) and Ashiu, Kyoto ($35^{\circ}18'N$, $135^{\circ}43'E$; 450 m above sea level), in central Japan. The forest in Takedao was dominated by *Quercus glauca* and *Q. serrata* (Fabaceae). The forest in Ashiu was dominated by *Cryptomeria japonica* (Cupressaceae), *Q. crispula*, *Q. serrata* and *Q. salicina* (Group for the Study on Ecology of Natural Forest, 1972).

2.2 | Observations and sampling of flower visitors

The diurnal and nocturnal pollinators were observed between late March and early April, from 2016 to 2019

and from 2017 to 2019, in Takedao and Ashiu, respectively. A female or male tree of *S. praecox* was selected from approximately 10 flowering trees and watched carefully during each observation. Diurnal observations were performed on sunny days between 9:00 a.m. and 5:00 p.m., at temperatures of 4–21°C, whereas nocturnal observations were performed for 4 h after sunset (start time: 6:00–6:33 p.m.) at temperatures of 4–14°C. If no flower visitors were observed throughout the diurnal observations or within 1 h after sunset during the nocturnal observations, the data were excluded from the study as this was an indication that the weather conditions may have been inappropriate for pollinator activity. Our preliminary observations revealed that most of the nocturnal flower visitors were active on flowers within 1 h after sunset.

The number of insects visiting inflorescences (height 1.5–3.0 m) on a *S. praecox* tree was recorded during each observation. The numbers of flowers observed on the female and male plants were 286.0 ± 131.6 (mean $\pm$ SD, $n = 5$) and 741.0 ± 809.5 ($n = 7$) in Ashiu, and 407.4 ± 152.3 ($n = 5$) and 418.8 ± 122.7 ($n = 5$) in Takedao, respectively. In Ashiu, the diurnal and nocturnal observations were conducted for 48 h and 24 h in total, respectively, whereas in Takedao, they were conducted for 32 h and 24 h in total, respectively.

Flower visitors to *S. praecox* were categorized into eight functional groups based on their morphology and behavior on the flowers, referring to previous investigations (Fenster, Armbruster, Wilson, Dudash, & Thomson, 2004; Gómez et al., 2008; Jürgens et al., 2014; Nikkeshi, Kurimoto, & Ushimaru, 2015). These included the following: (a) eusocial bees: *Bombus* spp. and *Apis cerana japonica* (Hymenoptera: Apidae); (b) small bees: Andrenidae, Apidae (*Ceratina* and *Nomada*), and Halictidae (Hymenoptera); (c) hoverflies: Syrphidae (Diptera); (d) bee flies: a long-tongued fly *Bombylius major* (Diptera: Bombyliidae); (e) other flies: short-tongued flies mainly from Empididae and Bibionidae (Diptera); (f) diurnal butterflies and moths: Lycaenidae and Callidulidae (*Pterodecta felderi*) (Lepidoptera); (g) nocturnal settling moths: Noctuidae, Geometridae, and Drepanidae (Lepidoptera); and (h) other insects: occasional visitors including wasps (Hymenoptera), beetles (Coleoptera) and others.

Visitors to the *S. praecox* flowers were randomly sampled using an insect net, and the proboscis lengths of the flower visitors were measured to the nearest 0.1 mm, with the aid of a slide caliper or with an ocular micrometer, under a stereomicroscope in the laboratory. The number of pollen grains of *S. praecox* on each flower visitor was counted using a stereomicroscope and categorized as 0, 1–10, 11–100 and > 100, in accordance with

previous investigations (Funamoto & Sugiura, 2016). Fisher's exact test was used to make comparisons between the diurnal flower visitors and the nocturnal settling moths (all collected from the female flowers) regarding the proportions of insects with *S. praecox* pollen grains. Data across sites and years were pooled for Fisher's exact test.

The number of flower visitors during diurnal and nocturnal observations was compared. The total number of insects that visited flowers during the peak activity time (diurnal, 4 h after noon; nocturnal, 4 h after sunset) was compared between diurnal and nocturnal observations using a generalized linear model (GLM), with a quasi-Poisson error distribution and log link. The total number of visitors per observation was used as a response variable. The period of observation (i.e., diurnal or nocturnal) and plant sex (i.e., male or female) were fixed factors. The number of visitors on female flowers or during the day was used as a reference. Data across the sites and years were pooled.

2.3 | Pollination experiments

To evaluate the relative contributions of nocturnal and diurnal pollinators to the female reproductive success of *S. praecox*, four pollination experiments were conducted in Ashiu, in 2019. Twenty-four inflorescences were randomly selected from three female trees for the following experiments, and three flower buds per inflorescence were used to make a total of 18 flowers for each of the four experiments. The experiments included: (a) natural pollination experiments, in which the female flowers were exposed to pollinators throughout anthesis; (b) cross-pollination experiments, in which female flowers were pollinated by pollen grains collected from the male plants and bagged throughout anthesis to exclude pollinators; (c) diurnal-pollination experiments, in which the female flowers were bagged from sunset to sunrise, to expose them to only diurnal pollinators for a week after anthesis, and then kept bagged until the end of the experiment; and (d) nocturnal-pollination experiments, in which female flowers were bagged from sunrise to sunset, to expose them to only nocturnal pollinators for a week after anthesis, and then kept bagged until the end of the experiment. Fine-mesh bags were used for bagging all the inflorescences. Selective exclusions of diurnal or nocturnal pollinators were conducted for a 1-week period. The selected flowers were checked approximately 2 months after the experiments, the presence or absence of fruits was recorded, and the number of seeds produced from the fruits was counted. One cross-pollinated flower was lost during the experiment. When a flower did not set fruit, the seed set was recorded as zero.

The number of seeds produced per flower was compared between the experiments using a GLM, with a quasi-Poisson error distribution and log link. The number of seeds produced per flower was used as a response variable. The types of pollination experiments used were fixed factors. Pairwise comparisons between experiments (e.g., diurnal pollination vs. nocturnal pollination) were conducted. The *p*-values were adjusted for multiple comparisons with false discovery rate-controlling procedures (Benjamini & Hochberg, 1995). All statistical analyses were performed using R version 3.5.2 (R Core Team, 2018).

3 | RESULTS

3.1 | Flower visitors

Stachyurus praecox had both diurnal and nocturnal flower visitors. Hymenopteran and dipteran insects were the main visitors during the day (Figures 1b–e and 2, Table S1). Small bees were the most frequent flower visitors during the day at both sites across the study years (except for a female in 2019 and a male in 2018 at Takedao; Figure 2 and Table S1). Small bees, hoverflies, bee flies (*Bombylius major*) and other flies (mainly Bibionidae and Empididae) visited the flowers for both pollen and nectar. Visitations by eusocial bees such as *Bombus* and *Apis* were infrequent. *Apis* were infrequent at both sites, and only one visit by *A. cerana japonica* was observed in 2017 at Takedao. Visitations by the bee flies and diurnal butterflies and moths were observed only at Ashiu. Settling moths (Noctuidae, Geometridae and Drepanidae) visited the flowers for nectar and were exclusive nocturnal visitors at both sites across the years (Figures 1f–h and 2, Table S1). Settling moths carried pollen grains of *S. praecox* on their probosces (Figure 1h), whereas the small bees and hoverflies carried *S. praecox* pollen on various parts of their bodies, including the head, thorax and legs (Figure 1b,e).

The number of diurnal flower visitors was higher than the number of nocturnal flower visitors (Figure S1). The number of visits per observation was influenced by the observation period (GLM, $t = -2.933$, $p = .009$), but not by the plant sex ($t = 1.351$, $p = .192$).

Pollen grains of *S. praecox* were found on the bodies of the flower visitors collected from both male and female flowers at both study sites (Table 1, Table S1). The proportion of insects with pollen grains of *S. praecox* was not significantly different between the diurnal and nocturnal visitors that visited the female flowers (Table 1; diurnal, 78.3%, $n = 23$; nocturnal, 59.0%, $n = 39$; Fisher's exact test, $p = .1673$). Pollen grains of *S. praecox* were found in

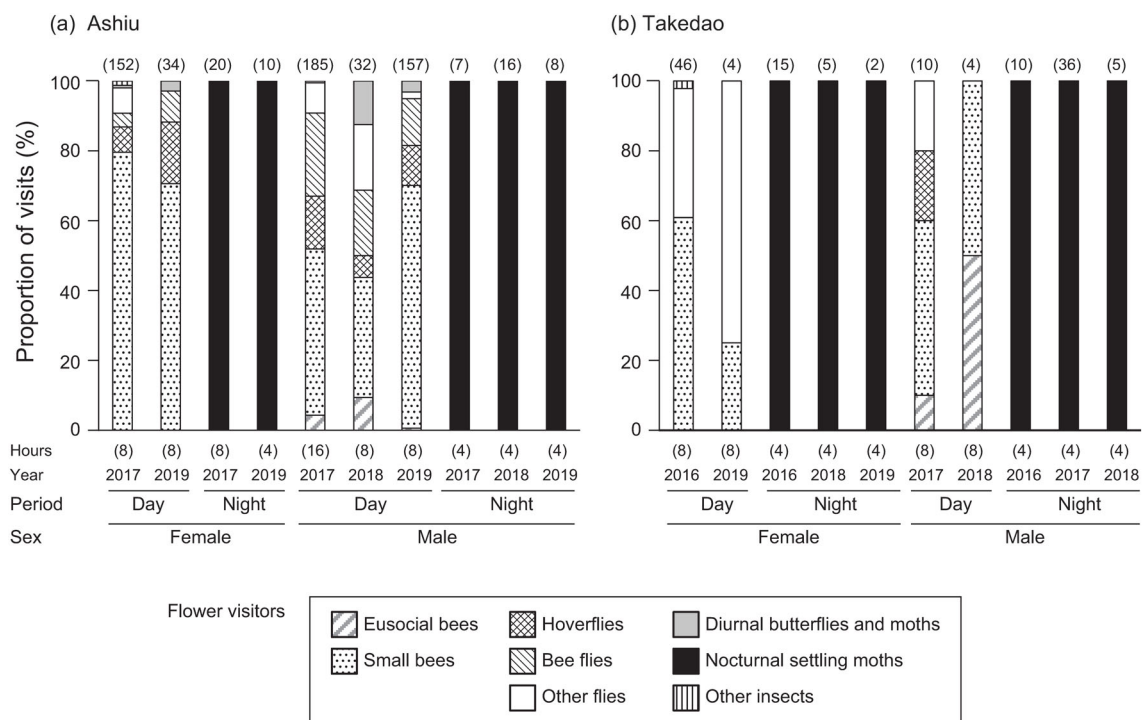


FIGURE 2 Proportion of visits by diurnal and nocturnal flower visitors of *Stachyurus praecox* in (a) Ashiu and (b) Takedao. The numbers in parentheses below each bar indicate the total number of hours of observations. The numbers in parentheses above the bars indicate the total number of visits during the observations

TABLE 1 Pollen load of the visitors collected from male and female flowers

			Number of flower visitors				Total
			<i>Stachyurus</i> pollen grains ^a				
			0	1–10	11–100	>100	
Plant sex	Period	Visitor functional group					
Female	Day	Bee flies	0	0	1	0	1
		Hoverflies	0	0	0	1	1
		Other flies	2	0	2	3	7
		Small bees	3	0	5	6	14
		Day subtotal	5	0	8	10	23
	Night	Nocturnal settling moths	16	6	3	14	39
		Night subtotal	16	6	3	14	39
Male	Day	Bee flies	0	0	3	1	4
		Hoverflies	1	0	1	5	7
		Other flies	0	0	0	5	5
		Eusocial bees	0	0	0	3	3
		Small bees	0	1	0	16	17
		Diurnal butterflies and moths	0	0	1	0	1
		Day subtotal	1	1	5	30	37
	Night	Nocturnal settling moths	4	1	7	11	23
		Night subtotal	4	1	7	11	23

Note: Data across the sites and years were pooled. Note that this table does not include the data of all flower visitors, as all individuals could not be collected.

^aThe number of insects with 0, 1–10, 11–100 and > 100 pollen grains of *Stachyurus praecox*.

23 (59.0%) of the 39 nocturnal settling moth specimens collected on female flowers; six moths carried 1–10 pollen grains, three carried 11–100 grains and 14 carried >100 grains (Table 1). Pollen grains of *S. praecox* were found on 11 of the 14 small bee specimens that were collected on female flowers; five small bees carried 11–100 grains and six small bees carried >100 grains (Table 1).

3.2 | Pollination experiments

The results of the pollination experiments showed that *S. praecox* was pollinated by both diurnal and nocturnal pollinators (Figure 3). However, the number of seeds produced in the nocturnal pollination experiment was significantly lower than that in the diurnal pollination experiment (GLM, $t = -3.828$, $p = .001$, adjusted $p = .002$), whereas the number of seeds produced in the diurnal pollination experiment was not significantly different from that in the cross-pollination experiment (GLM, $t = -0.525$, $p = .603$, adjusted $p = .603$). The

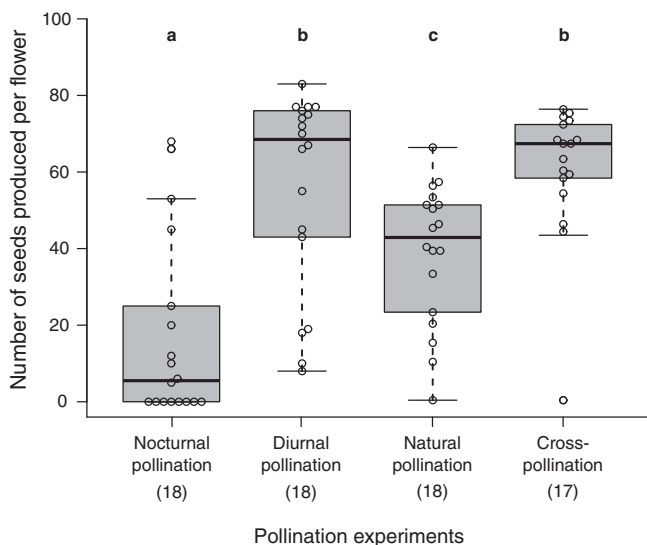


FIGURE 3 Number of seeds produced per female flower in each pollination experiment. Thick horizontal lines within the boxes show the median. The upper and lower boxes show the 75th and 25th percentiles, respectively. Whiskers show values within the 1.5 interquartile range. Circles indicate individual flowers. Different letters indicate significant differences among the experiments (GLM, $p < .05$). Pollination experiments: nocturnal pollination, flowers were bagged from sunrise to sunset so that they were exposed only to nocturnal pollinators; diurnal pollination, flowers were bagged from sunset to sunrise so that they were only exposed to diurnal pollinators; natural pollination, flowers were exposed to pollinators throughout anthesis; cross-pollination, flowers were pollinated by pollen grains collected from the male plants, and bagged throughout anthesis to exclude pollinators. The numbers in parentheses indicate the sample size

natural pollination experiment produced significantly fewer seeds than the diurnal pollination experiment ($t = -2.395$, $p = .022$, adjusted $p = .027$) and cross-pollination ($t = -3.407$, $p = .002$, adjusted $p = .003$).

4 | DISCUSSION

This investigation showed that *S. praecox* has a generalized pollination system that includes both diurnal and nocturnal pollinators. The most frequent diurnal flower visitors of *S. praecox* were small bees, whereas the settling moths were exclusive nocturnal flower visitors. This observation was consistent across both sites and years and is supported by previous investigations (Abe, 2007; Ikenoue, 1990; Ikenoue & Kanai, 2010) that have reported similar visitor groups to *S. praecox* flowers. Owing to the comparable seed production recorded for the diurnal-pollinated and cross-pollinated flowers, it is suggested that diurnal pollinators are more important than nocturnal ones in the pollination of *S. praecox*. Diurnal pollination and cross-pollination resulted in higher seed production when compared with natural pollination. However, this could be attributed to the fact that the naturally pollinated flowers were not bagged throughout the experiment; therefore, they were exposed to the harsh influences of abiotic and biotic factors, which could result in lower seed yields.

The selective exclusion experiments for diurnal and nocturnal pollinators showed that nocturnal settling moths partially contribute to the pollination of *S. praecox*. Previous investigations have suggested that nocturnal settling moths are incapable of pollinating during early spring (Alonso, 2004; Jürgens et al., 2014). The reports by Jürgens et al. (2014) and Alonso (2004) indicated that nocturnal moths have no contribution to the seed production of the two early spring flowering plants, *Salix caprea* and *Daphne laureola*, respectively. To the best of our knowledge, the current study provides the first experimental evidence that nocturnal moths contribute to the pollination of plant species that flower in early spring. Pollen grains of *S. praecox* were found on the probosces of the settling moths, indicating that pollination could have occurred when the settling moths visited the flowers for nectar. Hawkmoths are generally presumed to be the most important nocturnal moth pollinator (Hahn & Brühl, 2016). However, in the current study, the nocturnal pollinators were found to be settling moths, exclusively, and this is because only a few species of nocturnal hawkmoths are active in the early spring in this study region (Yano & Kishida, 2011).

The fact that *S. praecox* has a generalized pollination system involving both diurnal and nocturnal pollinators

might give it an advantage in early spring when pollinator activity is unpredictable. The results from the current study indicate that diurnal pollinators are more important than the nocturnal pollinators of *S. praecox* at Ashiu in 2019. This finding could be partly explained by the greater abundance of diurnal flower visitors than nocturnal visitors in this pollination system. However, the availability of small bees, the most frequent diurnal flower visitors of *S. praecox*, might be unpredictable for several factors. Firstly, small bees forage only on warm, sunny days, as they are ectothermic (Bishop & Armbruster, 1999; Herrera, 1995). According to Herrera (1995), no foraging activity of *Andrena bicolor* (Hymenoptera: Andrenidae) was observed on the flowers of *Narcissus longispathus* (Amaryllidaceae) when the ambient temperature was $<13^{\circ}\text{C}$. In contrast, the noctuid moths are capable of flying at ambient temperatures of $<10^{\circ}\text{C}$, as they are endothermic (Heinrich, 1987). Further studies are required to reveal the effect of ambient temperature on the activity of diurnal and nocturnal flower visitors, and the consequences for pollination success of early spring flowering plants. Secondly, several studies have shown that the onset of flowering in early spring plants tends to be earlier than the emergence of bee pollinators in warmer springs (Kehrberger & Holzschuh, 2019; Kudo & Cooper, 2019). Such a phenological mismatch between bee emergence and flowering might result in limitation of pollination of early spring plants in warmer springs (Kudo & Cooper, 2019; Kudo, Nishikawa, Kasagi, & Kosuge, 2004). In contrast, the nocturnal settling moths that visit early spring flowers emerge ahead of flowering; they reportedly visit molasses before *S. praecox* blooms (Ikenoue, 1990). Thus, the warming spring might not seriously disrupt the interactions between nocturnal settling moths and early spring flowers. Therefore, nocturnal settling moths could play a complementary role in pollination in early spring when the availability of diurnal pollinators is limited.

5 | CONCLUSION

This study showed that *S. praecox* has a generalized pollination system involving both diurnal and nocturnal pollinators. Although diurnal insects are the most important pollinators in *S. praecox*, nocturnal settling moths also contribute to the pollination of *S. praecox* at low temperatures in early spring. Further studies are required to reveal the relative roles of diurnal and nocturnal pollinators in different sites and years in *S. praecox*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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