Studies on pollinator trapping and rapid flower closure of carnivorous plants, Drosera spp.

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https://doi.org/10.15017/1931743

出版情報：九州大学, 2017, 博士（理学）, 課程博士
バージョン：
権利関係：

Studies on pollinator trapping and rapid flower closure of carnivorous plants, *Drosera* spp.

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Acknowledgements

I would like to express my gratitude to Dr. Tetsukazu Yahara for his insightful comments and suggestions throughout the study. I would like to thank Dr. Mikio Watanabe for his kind support in the field survey and giving me constructive comments through out the study. I would like to thank Dr. Eiiti Kasuya, Dr. Kazuhiro Eguchi, Dr. Shun K Hirota, Dr. Wu Fu-Yu, Dr. Shuichiro Tagane, Dr. Toru Nakahara, Mr. Kengo Fuse, Mr. Ryosuke Imasaka, Mr. Hironobu Kanemitsu and Ms. Haruka Osaki for helpful suggestions and comments for conducting the study. I thank Mr. Tadashi Minamitani and Mr. Shojiro Matsuura in the Study Group of Plants in Miyazaki Pref., Mr. Masumi Owada, and Ms. Sora Morimoto, Mr. Takuya Sato and Mr. Koki Kimura in Aichi University of Education for their kind support for conducting the field surveys. I also thank Dr. Firouzeh Javadi for correcting the English. I would like to thank the Board of Education in Kawaminami Town and Toyoake City for the permission of the surveys in the protected areas. Finally, I thank all the members of the ecological science laboratory of Kyushu University for their constructive comments and warm encouragements.

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Chapter 1. Pollinator trapping in selfing carnivorous plant species, *Drosera makinoi* and *D. toyoakensis* (Droseraceae)

Abstract

Carnivorous plants use insects not only as prey, but also as pollinators. Whereas outcrossing carnivorous plants are known to avoid trapping pollinators, selfing carnivorous plants may capture the pollinators as prey. Here, we provide evidence that two selfing carnivorous plant species with short flower-trap separation, *Drosera makinoi* (white-colored flowers) and *D. toyoakensis* (pink-colored flowers), caught some major pollinator species belonging to Diptera and Hymenoptera: four out of five species in *D. makinoi* and one out of six species in *D. toyoakensis*. We also tested the function of flowers to attract pollinator or prey insects by experimentally removing *Drosera* flowers. Flower removal did not significantly affect the number of trapped insects. On the other hand, the removal of flowers of co-flowering neighbor plants, *Eriocaulon decemflorum* for *D. makinoi* and *Lysimachia fortunei* for *D. toyoakensis,* significantly decreased the number of trapped insects. This finding suggests an exploitative relationship between *Drosera* spp. and co-flowering species.

Ecological Research (2018) 33: 487–494 DOI 10.1007/s11284-018-1572-6

Introduction

Carnivorous plants use specialized traps to catch insects and absorb nutrients from their bodies. This unique feeding habit has been considered as an adaptation to nutrient-poor habitats because carnivorous plants can fill deficiency of nutrients in soil by digesting trapped insects (Thum 1988; Zamora et al. 1997; Thoren and Karlsson 1998). Probably due to this advantage, carnivory evolved at least six times independently in the angiosperms (Ellison and Gotelli 2001). Catching insects is, however, not always advantageous for carnivorous plants because insects may serve as both prey and pollinators (Ellison and Gotelli 2001). If the same insect species serves as both, catching pollinators may decrease pollination success. Most carnivorous plant species may avoid this Pollinator-Prey Overlap (PPO) by making traps functional only after a flowering season (temporal separation) or placing traps apart from flowers (spatial separation) (Jürgens et al. 2012). The degree of PPO has been studied in some sundew species (*Drosera* spp.) in which traps are arranged in a rosette close to the ground, whereas their flowers are located high at the top of erect stems. For *D. pauciflora*, Anderson (2010) demonstrated that no pollinators were caught in traps. Murza et al. (2006) showed that basal traps of another sundew *D. anglica* caught a minor pollinator species *Thrips* sp. (Murza et al. 2006). However, it comprised only 3% of all flower visitors and major pollinators (Diptera, 95% of the total) were not caught by the traps of *D. anglica*.

On the other hand, high PPO may be observed in highly selfing carnivorous species in which trapping pollinators may not reduce fitness under negligible inbreeding depression (Sciligo 2009; Jürgens et al. 2012). Sciligo (2009) tested this idea using autonomously selfing *D. arcturi* that showed no inbreeding depression, and showed that 96% of pollinator fly families were trapped. To test the expectation of high PPO in highly selfing species, further studies on other *Drosera* species are needed (Jürgens et al. 2012). Here, we examine this expectation in two selfing annuals *Drosera makinoi* Masamune and *D. toyoakensis* M. Watanabe in which the inbreeding coefficient is 0.497 and 0.260, respectively (Watanabe, unpublished), indicating that selfing rate is higher in *D. makinoi.* In *D. makinoi* and *D. toyoakensis*, flowers are close to trap leaves arising from flowering stems and pollinators may approach trap leaves more frequently.

We also examine whether the flowers of two *Drosera* species attract pollinator and prey insects. Because two species are moderately inbreeding, it may be unnecessary to attract many pollinators required for high outcrossing. On the other hand, it may be advantageous to attract more insects by flowers as prey (Zamora 1999; Salces-Castellano et al. 2016). To test how the flowers are attractive to pollinators and prey insects, we experimentally removed flowers of *Drosera* spp. In addition, we also experimentally removed flowers of co-flowering neighbor species because those could also increase the number of insects trapped by the two *Drosera* species.

Specific questions addressed in this paper are as follows. (1) How large is the degree of PPO in two selfing carnivorous species, *D. makinoi* and *D. toyoakensis*? (2) Does the experimental removal of the flowers of *Drosera* spp. decrease the numbers of insects trapped by *Drosera* spp.? (3) Does the experimental removal of the flowers of co-flowering neighbor plants decrease the number of insects trapped by *Drosera* spp.?

Materials and Methods

Study species and sites

Drosera makinoi Masamune and *D. toyoakensis* M. Watanabe, previously included in *D. indica* (Watanabe et al. 2013; Kagawa 2015), are both annual plants with a single stem growing up to 10 to 20 cm. Linear and alternate trap leaves 5 to 7 cm long are scattered along the stem and trap leaves at the upper position are close to flowers (Fig. 1 (a)). Traps have glandular hairs called tentacles and glands secrete sticky liquid and digestive enzyme (Sun et al. 2014). The two species are different in flower color: white in *D. makinoi* and pink in *D. toyoakensis*. Both *Drosera* species grow in open bogs of a few restricted localities and have been listed as threatened plants (Japan Wildlife 2015). *Drosera makinoi* has a scattered distribution in Japan and the distribution of *D. toyoakensis* is now restricted to Aichi Prefecture, Japan. *Drosera toyoakensis* germinates early in May and has three to four flowers at the top of the stem from July to September. Flowers open around 8 am and close by 1 pm. A flower blooms only once, and it does not open again after closing even if closing is due to strong rain or wind. About 500 seeds per flower mature in the middle of September, and the plants begin to wither in the middle of October. *Drosera makinoi* has similar phenology but a longer flowering period from July to October. We made observations and field experiments of *D. makinoi* in a *Sphagnum* bog at Watarase (Tochigi City, Tochigi Pref., Japan). The bog is about 200 m^2 and neighboring to a population of common reed *Phragmites australis* (Cav.) Trin. ex Steud. (Poaceae). Studies of *D. toyoakensis* were carried out in a *Sphagnum* bog at

Toyoake (N 35°4'26" E 137°1'7", Toyoake City, Aichi Pref., Japan). The bog of about 200 m^2 is surrounded by a protection fence and is neighboring to paddy fields.

Flower visitor observations

To determine whether pollinator species were trapped by *D. makinoi* and *D. toyoakensis*, we observed flower visitors from August to September for three years (2012-2014). For *D. makinoi*, we observed pollinators from 8 am to 2 pm, and for *D. toyoakensis*, from 7 am to 2 pm while careful not to damage the bog. We observed each flower visitor until it visited five flowers of *Drosera* or it flew away before visiting five flowers, and recorded the taxonomic identity of insect species and whether it touched stamens and/or pistils. Some flower visitors of *D. makinoi* and *D. toyoakensis* visited *Eriocaulon decemflorum* Maxim. (Eriocaulaceae) and *Lysimachia fortunei* Maxim. (Primulaceae), respectively (Fig. 1 (b), (c)). The plant species was recorded if the flower visitor moved from *Drosera* species to another species (co-flowering neighbor species) and vice versa. A pollinator was defined as an individual that (1) touched a stamen and/or a pistil and (2) carried pollen grains. Flower visitors were caught with a butterfly net and fixed with 95% ethanol. Later in the laboratory, the entire body of each insect visitor was stained with 45% aceto-carmine solution and observed under a stereomicroscope to check whether it carried pollen grains. Based on results from the flower-visitor survey and the following prey survey, we calculated an index of pollinator-prey species overlap J as the number of trapped pollinator species divided by the total number of pollinator species; J =

0 when no pollinator species was trapped, while $J = 1$ when all pollinator species were trapped.

We computed all statistical analyses using R 3.1 (R Core Team 2010). To examine whether there is a difference in the number of flower visits between *Drosera* and co-flowering neighbor plant species, we used a generalized linear mixed model (GLMM) with the plant species, pollinator species, and the interaction term as explanatory variables, and survey date as a random factor. At first, we verified the significance of the interaction term by comparing the model with and without the interaction term. In Watarase, we adopted the model without the interaction term because the effect of the interaction was not significant. In Toyoake, the interaction term was significant because some pollinators visited *Drosera* flowers more frequently than flowers of neighbor plant species, and others visited flowers of neighbor plant species more frequently than *Drosera* flowers. Thus, we only used the data of pollinator species that visited flowers of both *Drosera* and neighbor plant species at least once in our survey. We used a likelihood-ratio test (type II) to determine any significant effect of explanatory variables. These GLMM analyses were performed with a log link and a Poisson distribution (Crawley 2005) using the R package “lme4” (Douglas et al. 2015). To describe the degree of pollinator sharing between *Drosera* and co-flowering neighbor plant species, we calculated the niche overlap index (NOI) (Pianka 1973); the index ranges from 0 (no overlap) to 1 (complete overlap) using the R package “EcoSimR” (Gotelli et al. 2015).

Flower removal experiments

To determine the effect of flowers of *Drosera* and neighbor plants (*E. decemflorum* for *D. makinoi* and *L. fortunei* for *D. toyoakensis*) on the number of insects trapped by *D. makinoi* or *D. toyoakensis*, we removed flowers of *D. makinoi*, *D. toyoakensis* and neighbor plants during the flowering periods at study sites of *D. makinoi* and *D. toyoakensis*: September 5 and 6, 2014 for *D. makinoi* and September 2 and 3, 2014 for *D. toyoakensis*. For each species, we set the following four classes of 40 cm × 40 cm quadrats at an interval of 40 cm or longer: (1) a treatment where flowers of both *Drosera* species and neighbor species were left, (2) a treatment where flowers of *Drosera* species were left but flowers of neighbor plants were removed, (3) a treatment where flowers of neighbor plants were left but flowers of *Drosera* species were removed, and (4) a treatment where flowers of both *Drosera* species and neighbor plants were removed. For each treatment, we set one quadrat containing 20 individuals of each *Drosera* species and co-flowering neighbor species. When the quadrat had more than 20 individuals, we cut flowers of excessive individuals to equalize the sample size; here, we regarded the sample size as 20 by considering a plant as a unit of replication. On each observation day (from 6 am to 7 am), we removed all flower buds of *Drosera* and/or neighbor plants that were just before flowering and we removed all dead insects on the surface of traps. For both *Drosera* species, we collected prey insects on traps from noon to 1 pm to identify species and recorded the number of prey individuals per day per plant in each quadrat. We then measured the body length of a prey insect, distance between each prey insect and the nearest flower, the presence/absence of flowers, and the height (from the ground) of traps where the prey was trapped.

To determine whether the flowers of *Drosera* species and neighbor species affected the number of prey individuals trapped, we used a generalized linear model (GLM) with the number of insects trapped per day per plant, the number of pollinators trapped per day per plant or the number of non-pollinators trapped per day per plant as the response variable. At first, the presence/absence of flowers of *Drosera*, the presence/absence of flowers of neighbor species and the interaction of these two variables were considered as the explanatory variables. Because the interaction effect on the response variable was not significant, we used a model without the interaction term to test effects of the two variables. Because the observed number of prey insects showed over-dispersion, we used a negative-binomial error distribution and a log link (Zuur et al. 2009) with a function “glm.nb” in the R package “MASS” (Venables and Ripley 2002).

Results

Pollinator-prey species overlap

In *D. makinoi*, five pollinator species of Diptera, Hymenoptera and Lepidoptera were recorded (Table 1 (a), Fig. 2 (a)) and all five pollinator species except *Mathias oberthueri* (Lepidoptera) were trapped as prey (Table 1 (a)). In *D. toyoakensis*, six pollinator species of Diptera and Lepidoptera were recorded (Table 1 (b), Fig. 2 (b)) among which only a syrphid fly *Sphaerophoria menthastri* (Diptera) was trapped as prey (Table 1 (b)). The index of pollinator-prey species overlap *J* was 0.83 ± 0.21 (mean ± SD, n = 5) in *D. makinoi* and 0.071 ± 0.18 (mean ± SD, n = 6) in *D. toyoakensis*; it was positive in every month in *D. makinoi*, but positive only in September 2014 in *D. toyoakensis*.

A syrphid fly (*S. menthastri*, Fig. 3) was the most frequent visitor to flowers of *D. makinoi* (22.6% of all flower visits; mean ± SD = 1.3 ± 1.8) and *D. toyoakensis* (67.3% of all flower visits; mean ± SD = 3.3 ± 7.3). *Sphaerophoria menthastri* carried 4.6 ± 3.2 (mean ± SD, n = 5) pollen grains of *D. makinoi* and 14 ± 8.2 (mean ± SD, n = 5) pollen grains of *D. toyoakensis*.

Four pollinator species visited *E. decemflorum*, a co-flowering neighbor species of *D. makinoi*. All four species were overlapped with pollinator species of *D. makinoi* and three of four species were overlapped with prey species of *D. makinoi* (Table 1 (a)). *Drosera makinoi* and *E. decemflorum* showed a large overlap of their pollinator species (NOI = 0.761). There was no significant difference between the number of flower visits in *D. makinoi* and *E. decemflorum* (Fig. 4 (a), GLMM *p* = 0.730). Seven pollinator species visited *L. fortunei*, a co-flowering neighbor species of *D. toyoakensis*. Four out of seven species were overlapped with pollinator species of *D. toyoakensis* and one of seven species was overlapped with prey species of *D. toyoakensis* (Table 1 (b)). *Drosera toyoakensis* and *L. fortunei* showed a large overlap of their pollinators (NOI = 0.834). The effect of the interaction of plant species and pollinator species on the number of flower visits was significant (GLMM $p < 0.001$), and some pollinator species visited *D. toyoakensis* more frequently (e.g. *Eurema hecabe*, 5.5 ± 5.5 times on *D. toyoakensis*, 2.5 ± 0.50 times on *L. fortunei*) while *S. menthastri* visited *L. fortunei* more frequently (4.5 ± 7.6 times on *D. toyoakensis*, 9.7 ± 8.5 times on *L. fortunei*). The number of flower visits by pollinators was significantly higher in *L. fortunei* than in *D. toyoakensis* when we used the data of pollinator species that visited *D. toyoakensis* and *L. fortunei* at least once in our survey (Fig. 4 (b), GLMM Estimate ± SE = 1.8 ± 0.82, $p < 0.001$).

Flower removal experiments

Flowers of *D. makinoi* had no significant effect on the number of insects trapped, whereas flowers of a co-flowering neighbor species *E. decemflorum* had a significant positive effect on the number of both pollinator and non-pollinator species trapped (Table 2 (a), Fig. 5 (a)-(c)). Flowers of *D. toyoakensis* had no significant effect on the number of insects trapped, whereas flowers of a co-flowering neighbor species *L. fortunei* had a significant positive effect on the number of pollinator species trapped (Table 2 (b), Fig. 5 (d)-(e)). Flowers of co-flowering neighbor species *L. fortunei* had no significant effect on the number of non-pollinator species trapped (Table 2 (b), Fig. 5 (f)).

Discussion

The two *Drosera* species trapped their pollinators, four of five pollinator species in *D. makinoi* and one of six species in *D. toyoakensis*, including syrphids that are known as effective pollinators of many flowering plants (Willmer 2011; Woodcock et al. 2014). This finding supported our prediction that selfing species may show large PPO. Because both *D. makinoi* and *D. toyoakensis* do not have mechanisms such as temporal, spatial, and chemical separation of traps from flowers (Jürgens et al. 2012; El-Sayed et al. 2016), flying pollinators are likely to be trapped by accident. A similar case is reported in a selfing species *D. arcturi*, which has flowers close to traps and catches major pollinator species (Sciligo 2009).

Whereas the two *Drosera* species trapped their pollinators, the experimental removal of flowers of neither *D. makinoi* nor *D. toyoakensis* significantly changed the number of insects trapped. This may be because autonomous selfing species like *D. makinoi* and *D. toyoakensis* allocate small amounts of resources to pollinator attraction (Barrett 2002). On the other hand, the experimental removal of flowers of co-flowering neighbor plants significantly reduced the number of pollinator insects trapped. This finding shows that the number of flowers of not *Drosera* but neighbor plants maintains the density of pollinator species, although the sample size is small and further tests using more quadrats are desirable. This positive relationship is similar to the relationship called pollination facilitation in pollination biology: the presentation of flowers by one species attracts pollinators to the other neighboring plant species (Feldman et al. 2004; Ghazoul 2006). Pollination facilitation can occur when two or more co-flowering plants form large and collective floral displays (Morales and Traveset 2009; Ye et al. 2014) that increase pollinator density in a patch. Facilitative pollination especially benefits species occurring at low densities (Schemske 1981; Morales and Traveset 2009) and rewardless species growing near rewarding species (Johnson et al. 2003). In our study, the existence of flowers of co-flowering plants benefits *Drosera* plants by increasing the density of prey insects in a patch. Therefore, *Drosera* plants may experience pollination facilitation in the presence of co-flowering neighbors. On the other hand, *Drosera* plants may impose a cost on co-flowering neighbors by decreasing the density of pollinators and pollination efficiency. Therefore, co-flowering neighbors may experience exploitation rather than facilitation.

Drosera makinoi trapped more pollinator species than *D. toyoakensis*. This difference may be explained by the difference of flower color: *D. makinoi* has white flowers, which are similar to neighboring *E. decemflorum*, whereas *D. toyoakensis* has pink flowers, which are not similar to neighboring *L. fortunei*. Corresponding to this difference, the frequency of flower visits was not significantly different between *D. makinoi* and *E. decemflorum* in all pollinator species, but was significantly different between *D. toyoakensis* and *L. fortunei* for some pollinator species. Under these circumstances, pollinator species visiting neighboring plant flowers are expected to be trapped more frequently in *D. makinoi*, because pollinators of *D. makinoi* probably share a similar search image to white flowers of *Drosera* and *L. fortunei*. Alternatively, it may be explained by the difference of selfing level between the two species: the inbreeding coefficient was higher in *D. makinoi* (*F* = 0.497) than in *D. toyoakensis* (*F* = 0.260)

(Watanabe, unpublished). Pollinator dependence is expected to be lower in the more highly selfing species *D. makinoi* in which trapping pollinator species is considered to be more advantageous. This result may support the prediction that species with a higher selfing rate are likely to trap pollinators more frequently. For a more rigorous test of this prediction, however, further studies on the degree of PPO in plants with a wide range of selfing rates are needed.

In conclusion, by studying selfing species of *Drosera*, we showed evidence of trapping major pollinator species in carnivorous plants that were attracted by flowers of neighbor plants. This finding suggests that *Drosera* plants are exploiting pollinator resources of co-flowering neighbor species. We suggest that selfing carnivorous species provide a unique opportunity to test various ideas on pollinator-prey relationships in carnivorous plants. More species of selfing carnivorous plants are known in *Drosera* and other carnivorous plants and further studies on those species would deepen our understanding on PPO as well as possible exploitative relationship between carnivorous species and co-flowering neighbor species.

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Figures and Tables

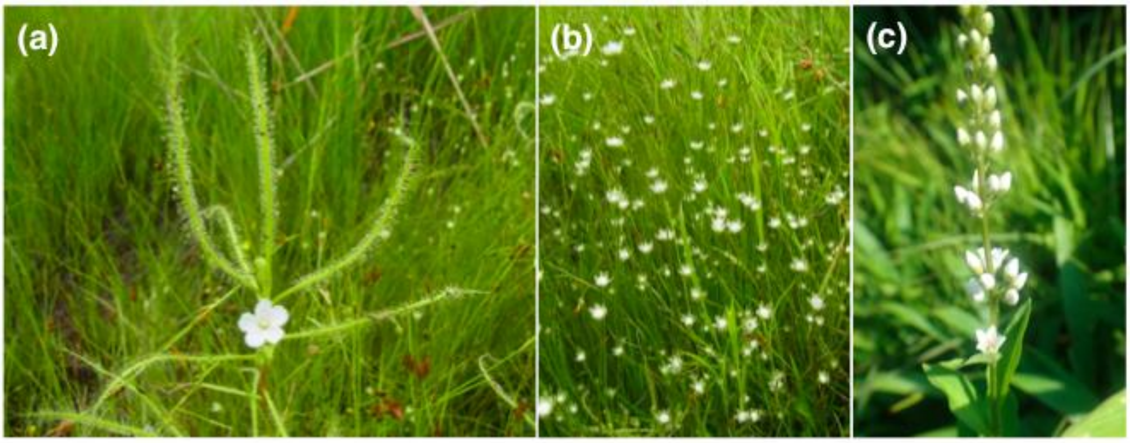

Fig. 1 *Drosera makinoi* and two co-flowering neighbors at the study sites of *Drosera spp*.

(a) *Drosera mainoi*. (b) *Eriocaulon decemflorum*, a co-flowering species growing with *D. makinoi* at Watarase site. (c) *Lysimachia fortunei*, a co-flowering species growing with *D. toyoakensis* at Toyoake site.

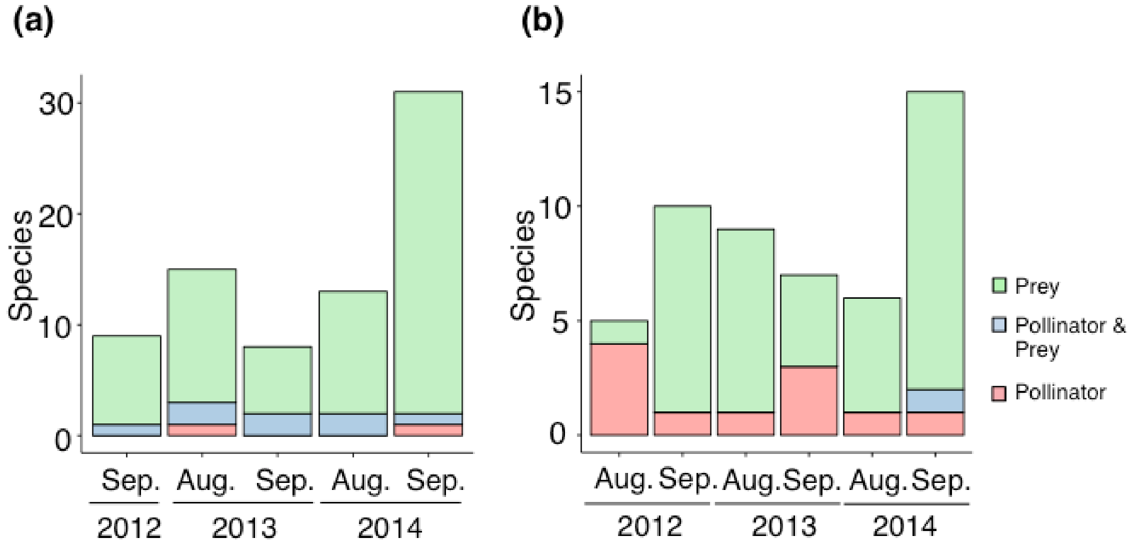

Fig. 2 Monthly variations of pollinator and prey species composition.

Green, blue and red bars respectively indicate the number of prey species that were not regarded as pollinators, the numbers of species regarded as both prey and pollinators, and the number of pollinator species not regarded as prey. (a) *Drosera makinoi*, (b) *D. toyoakensis*.

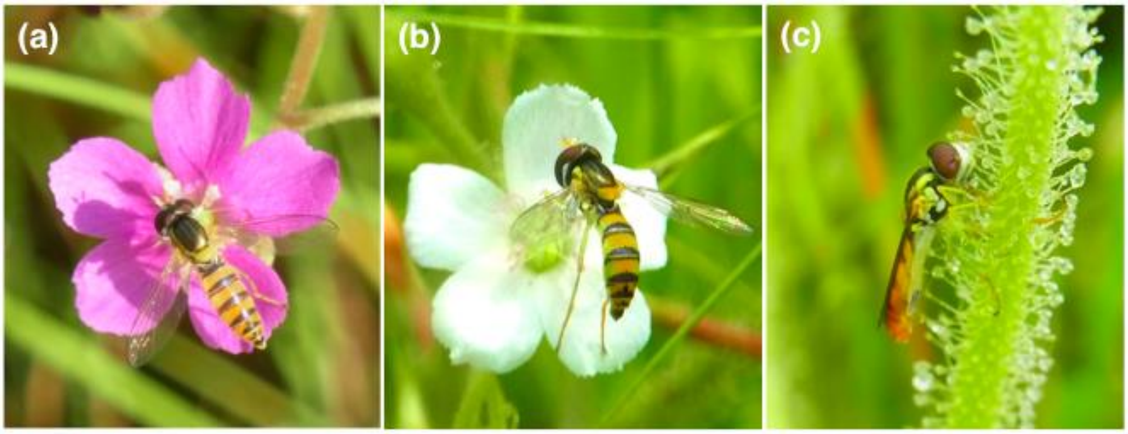

Fig. 3 The most frequent pollinator species: *Sphaerophoria menthastri*.

The hoverfly *S. menthastri* was a pollinator species common to *Drosera makinoi* and *D. toyoakensis*. (a) *S. menthastri* landing on a *D. toyoakensis* flower. (b) *S. menthastri* landing on a *D. makinoi* flower. (c) *S. menthastri* trapped by *D. makinoi* (also trapped in *D. toyoakensis,* not shown).

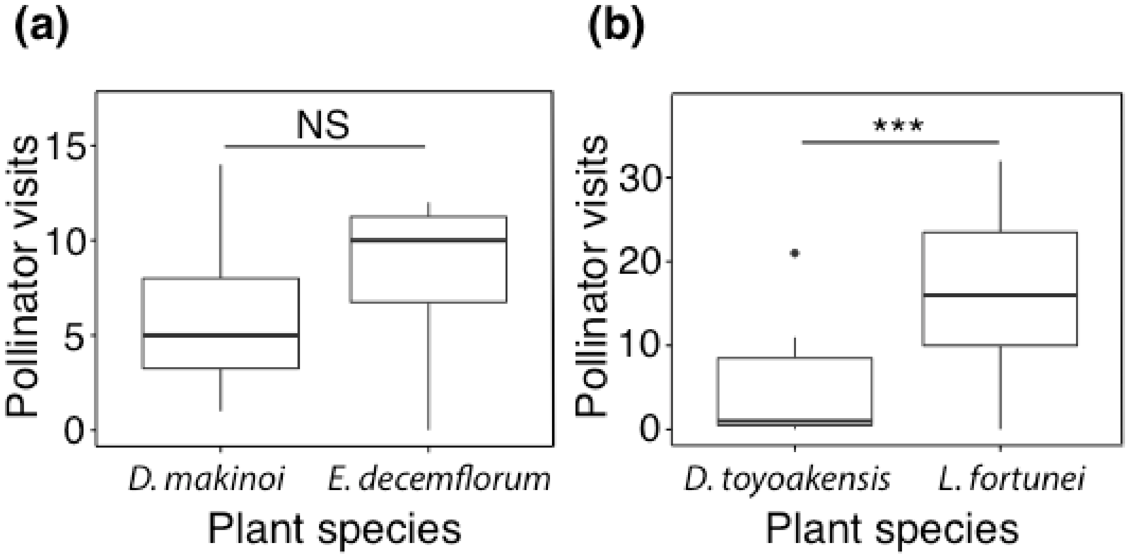

Fig. 4 Number of pollinator visits for *Drosera* and co-flowering neighbor species.

(a) There was no significant difference in pollinator visits between *D. makinoi* and a co-flowering neighbor species *Eriocaulon decemflorum* at the Watarase site (GLMM *p* = 0.73). (b) Pollinators visited a co-flowering neighbor species *Lysimachia fortunei* more frequently than *D. toyoakensis* at the Toyoake site (GLMM *p* < 0.001).

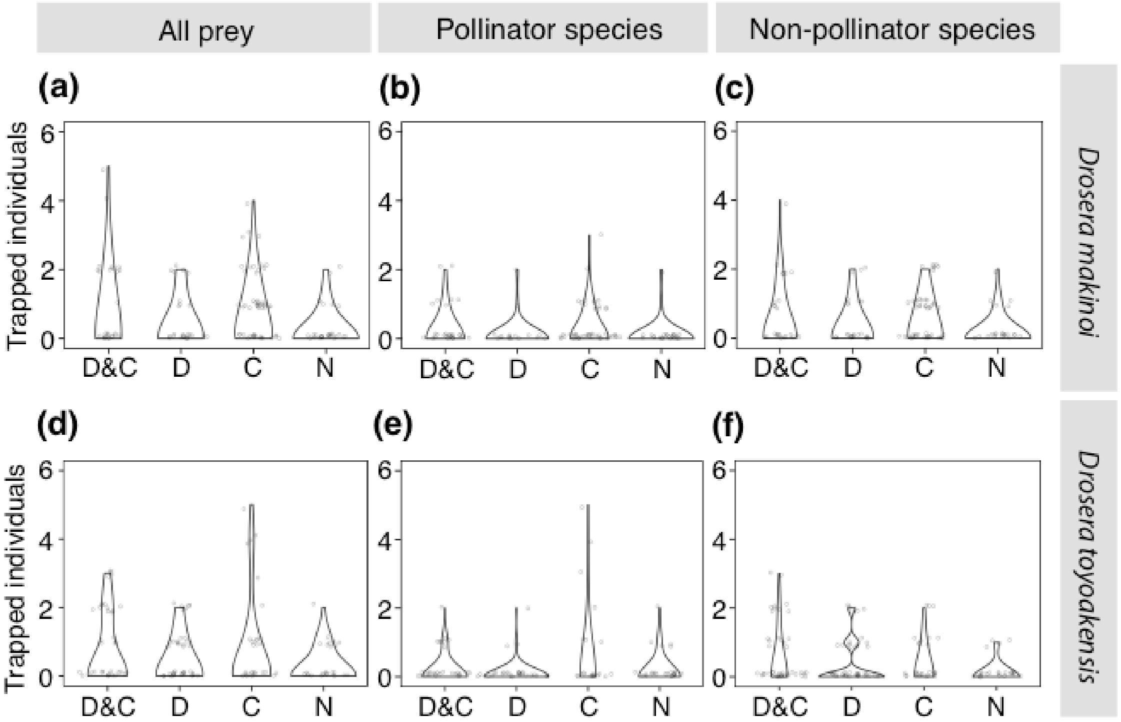

Fig. 5 Kernel density (black line) and observed value (gray circles) of trapped prey individuals on four treatments with the combination of presence / absence of *Drosera* and presence / absence of co-flowering neighbor plants flowers. D&C: Both *Drosera* and co-flowering neighbor plants, D: only *Drosera*, C: only co-flowering neighbor plants, N: Neither *Drosera* nor co-flowering plants existed. Flowers of neighbor plants positively affected the number of (a) all prey species and (b) pollinator species trapped by *Drosera makinoi* and (d) all prey species and (e) pollinator species trapped by *D. toyoakensis*. Flowers of neighbor plants positively affected the number of (c) non-pollinator species trapped by *D. makinoi,* while did not affect significantly the number of (f) non-pollinator species trapped by *D. toyoakensis*.

Table 1 Pollinator and prey species of *Drosera* and co-flowering neighbor species

(a) *Drosera makinoi* and *Eriocaulon decemflorum*

Pollinator species	*D. makinoi* flower visits (mean ± SD)	*E. decemflorum* flower visit (mean ± SD)	trapped (mean)
Mesembrius flaviceps	5.0 ± 4.0	4.5 ± 4.5	1.2
Sphaerophoria menthastri	3.3 ± 1.7	6.7 ± 4.8	1.4
Andrena sp.	0.50 ± 0.50	0.50 ± 0.50	0.2
Apis mellifera	3	0	0.13
Mathias oberthueri	1	1	0

(b) *D. toyoakensis* and *Lysimachia fortunei*

Pollinator species	*D. toyoakensis* flower visits (mean ± SD)	*L. fortunei* flower visits (mean ± SD)	trapped (mean)
Eupeodes corollae	0.25 ± 0.43	4.5 ± 4.0	0
Lucilia sp.	0	5	0
Paragus haemorrhous	0	3.0 ± 2.0	0
Sphaerophoria menthastri	4.5 ± 7.6	9.7 ± 8.5	2.5
Eurema hecabe	5.5 ± 5.5	2.5 ± 0.50	0
Lycaena phlaeas	0	10	0
Zizeeria mahargia	0.33 ± 0.47	5.3 ± 3.7	0

Table 2 Results of generalized linear model analyses on the effect of flowers of *Drosera* and co-flowering neighbor plants on (a) Watarase site (*Drosera makinoi* and *Eriocaulon decemflorum*) and (b) Toyoake site (*D. toyoakensis* and *Lysimachia fortunei*).

(a) Watarase site

Response variable: All prey individuals

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-1.2 ± 0.27	-4.6	**4.9 × 10^{-6}*****
Drosera makinoi	0.089 ± 0.26	0.35	0.73
Eriocaulon decemflorum	0.90 ± 0.28	3.2	**0.0015****

Response variable: Prey overlapped with pollinator species

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-2.5 ± 0.52	-4.8	**3.7 × 10^{-6}*****
Drosera makinoi	-0.097 ± 0.43	-0.23	0.82
Eriocaulon decemflorum	1.1 ± 0.54	2.1	**0.038***

Response variable: Prey not overlapped with pollinator species

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-1.6 ± 0.30	-5.3	**1.1 × 10^{-7}*****
Drosera makinoi	0.15 ± 0.27	0.53	0.60
Eriocaulon	0.81 ± 0.31	2.6	**0.0084****

decemflorum

(b) Toyoake site

Response variable: All prey individuals

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-1.4 ± 0.38	-3.6	**2.7 × 10^{-4}*****
Drosera toyoakensis	0.22 ± 0.37	0.61	0.54
Lysimachia fortunei	0.87 ± 0.37	2.3	**0.019***

Response variable: Prey overlapped with pollinator species

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-2.3 ± 0.60	-3.9	**1.0 × 10^{-4}*****
Drosera toyoakensis	0.020 ± 0.57	0.036	0.97
Lysimachia fortunei	1.2 ± 0.60	1.9	**0.050***

Response variable: Prey not overlapped with pollinator species

Explanatory variable	Estimate ± SE	*Z*	*P*
(Intercept)	-1.8 ± 0.44	-4.2	**2.7 × 10^{-7}*****
Drosera toyoakensis	0.32 ± 0.43	0.75	0.45
Lysimachia fortunei	0.66 ± 0.42	1.6	0.12

Chapter 2. A sensitive flower: mechanical stimulation induces rapid flower closure in *Drosera* spp. (Droseraceae)

Abstract

Some plants rapidly close its leaves in response to mechanical stimulation, but no case is known in which mechanical stimulation causes rapid petal closure. In this study, we found that *Drosera tokaiensis* closes petals within 2-10 min after experimental stimulation of calyx, closed flowers or scapes with a pair of tweezers. While petal closure was induced more rapidly by touching a position closer to a flower, it was not induced by stimulating stamens and pistils. The habit of petal closure varies among species of *Drosera*: by experimental stimulations of calyx or scapes, *D. tokaiensis* and *D. spatulata* often closed petals but *D. rotundifolia* and *D. toyoakensis* did not close them. The petal closure may function as defense against a specialist florivore.

Plant Species Biology (2018) doi: 10.1111/1442-1984.12203

Introduction

The rapid movement of plants induced by mechanical stimulation has attracted considerable attention by scientists since Darwin's time (Darwin 1880). One famous example is the "sensitive plants" (*Mimosa pudica*), which folds its leaflets using specialized motor organs at the bases of leaflets within 2 sec after mechanical stimulation (Campbell & Thomson 1977; Fromm 1991; Simons 1992), probably to deter herbivory (Eisner 1981; Braam 2004). Another example is a carnivorous plant Venus fly trap (*Dionaea muscipula*) that catches insects by closing its leaves using motor cells within 0.5 sec when insects touch small trigger hairs (Braam 2004; Volkov et al. 2007). Thirdly, another group of carnivorous plants the sundew (*Drosera* spp.) moves glandular hairs using motor cells within several seconds to some minutes after insects touch them (Poppinga et al. 2013).

Here, we report a novel case of rapid plant movement: rapid petal closure induced by mechanical stimulation of calyx or scapes in *Drosera* spp. In the fields, we accidentally found that *D. tokaiensis* closes petals within 2-10 min when we touched the scape by hand (Fig. 1). The purpose of this paper is to characterize this rapid petal closure by reporting the results of stimulation experiments on the scape and floral organs of *Drosera* spp.

Materials and Methods

Drosera tokaiensis is a perennial carnivorous plant species growing on bogs, having trap leaves in a rosette 2-4 cm across (Kagawa 2015). At the flowering stage, 1 to 2 flower scapes with a spike of 1 to 10 buds are stretched (flower scape: mean ± se = 16.9 ± 0.268 cm, n = 17). On the morning of a sunny day, only one flower per spike opens between 7:00 am to 8:00 am (Fig. 1a), and a flower (both petals and calyx) closes between 0:00 pm to 1:00 pm. Flowers on a spike show acropetal maturation and a closed flower that bloomed one day before (shown as "F" in Fig. 2) is located just below a flower at blooming.

We made field observations and experiments for *D. tokaiensis* on a sunny day of 24 July 2017 in Toyoake City, Aichi Pref., Japan (N 35.0739, E 137.0185). Before 7:50 am, we randomly selected blooming individuals by a computer program of random number generation with R (R Core Team 2010). Then we experimentally touched stamens and pistils (S&P, n = 4), calyx (C, n = 6), the top of a scape (TS, n = 4), a closed flower located just below the blooming flower (F, n = 4) and a middle position of a scape (MS, n = 3), with a pair of tweezers about 10 times for 10 sec between 7:50 am to 10:20 am. We recorded whether "flower closure" occurred or not as a binary state (0/1) at 2, 3, 5 and 10 min after stimulation. We defined "flower closure" as the situation where all stamens and pistils become completely hidden by petals. To record the movement of petals and calyx, we took photographs of flowers with a digital camera (Olympus Stylus TG-3) before giving stimulation and at 2, 3, and 5 min after the stimulation. Using an image processing program Image J (Schneider et al. 2012), we measured the angle between an outermost petal and the extended line of a flower stem (θ_{p}), and the angle between an outermost lobe of calyx and the extended line of flower stem (θ_{c}). We designated θ_{p} as “petal openness” and θ_{c} as “calyx openness.”

Using obtained data, we tested the effect of stimulated position on the proportion of flower closure using a generalized linear model (GLM) with the binary state (open or close) as a response variable, a binomial error distribution, a logit link function, and the two explanatory variables; stimulated position (S&P, C, TS, F or MS) and time of stimulation (lapsed second from 7:00 am). Type I error for multiple comparisons was adjusted by the Bonferroni-Holm method.

We also tested the effect of stimulated position on the speed of petal closure. To describe the speed of petal closure, we calculated “change of petal openness” (⊿θ_{p}) by subtracting θ_{p} at 2, 3, and 5 min after the stimulation from θ_{p} before stimulation. We used a linear mixed model (LMM) with ⊿θ_{p} as a response variable, the following five explanatory variables as fixed effects, (1) time after the stimulation (2, 3, 5 min), (2) time of stimulation (lapsed time in seconds from 7:00 am), (3) openness before mechanical stimulation, (4) class of stimulated position (near [S&P, C or TS] or far [MS] from petals), (5) the interaction term of time after the stimulation and class of stimulated position, and individual ID as a random effect. When the interaction term is significant, the slope of linear regression of ⊿θ_{p} on time after the stimulation differs significantly between two classes of stimulated position, indicating significant difference in the speed of petal closure. We tested differences of θ_{c} (calyx openness) at 2, 3, 5 min after stimulation using a LMM with θ_c as a response variable, the following two explanatory variables as fixed effects, (1) time after the stimulation (2, 3, 5 min), (2) time of stimulation (lapsed time in seconds from 7:00 am), and individual ID as a random effect.

To examine whether other species of *Drosera* also close petals in response to mechanical stimulation, we made field experiments on the following three species on sunny days; *D. spatulata*, in Kawaminami Town, Miyazaki Pref. (N 32.2041, E 131.5290) on 12 July 2017 and Uruma City, Okinawa Pref. (N 26.4479, E 127.8327) on 6 July 2017, *D. rotundifolia* in Karatsu City, Saga Pref. (N 33.3572, E 130.0679) on 22 July 2017, and *D. toyoakensis* in Toyoake City, Aichi Pref. (N 35.0739, E 137.0186) on 24 July 2017. We stimulated three positions (S&P, C and MS) and recorded whether flowers were closed or open after 10 min.

All statistical analyses were conducted with R (R Core Team 2010) using packages “car” (Fox & Weisberg 2011), “fmsb” (Nakazawa 2017), “ggplot2” (Wickham 2009), “lme4” (Douglas et al. 2015) and “lmerTest” (Kuznetsova et al. 2017).

Results

Stimulation of calyx (C), the top of a scape just below calyx (TS), a closed flower located just below the blooming flower (F), and the middle position of a scape (MS) induced petal closure irrespective of time of stimulation, but stimulation of stamens and pistils (S&P) did not induce petal closure. When we stimulated either of three positions near petals (C, TS, F), petals moved inward and concealed stamens and pistils within 2-5 min (mean ± se = 2.57 ± 0.01 min) (Fig. 2, video: https://www.youtube.com/watch?v=ls45d7DWFfA&feature=youtu.be). On the other hand, when we stimulated a position far from petals (MS), petals moved more slowly and concealed stamens and pistils within 5-10 min (mean ± se = 7.50 ± 1.88 min) (Fig. 2). There was a significant difference in the speed of petal closure between the classes of stimulated position (Table 1, Fig. 2b, LMM, the interaction of lapsed time and stimulated position: $P < 0.01$). Consequently, after 2 or 3 min, the proportion of flowers showing petal closure differed significantly between the classes of stimulated position (GLM, all combinations, $P < 0.05$), but it did not differ after 5 min (GLMs, all combinations, $P > 0.05$). Contrary to petals, calyx lobes did not close by stimulation; the degree of calyx openness (θ_c) did not change significantly in 5 min (Fig.1d, LMM, $P = 0.334$). Calix closed around noon, as well as petals without stimulation (Fig.1e).

Among the other three species, *D. spatulata* frequently (23/25) closed petals in response to stimulation on calyx and sometimes (5/14) closed in response to stimulation on a middle position of a scape, but *D. rotundifolia* and *D. toyoakensis* did not close petals in response to stimulation of either calyx or a middle position of a scape (Table 2).

Stimulation of stamens and pistils did not induce petal closure in the three species as in *D. tokaiensis*.

Discussion

Mechanical stimulation on calyx, closed flowers or scapes of *Drosera tokaiensis* induced petal closure in 2-10 min but did not induce calyx closure. Petal closure occurred faster when a position closer to the flower was stimulated. On the other hand, stimulation of neither stamens nor pistils induced petal closure. *Drosera spatulata* closed petals when calyx or scapes were stimulated but *D. rotundifolia* and *D. toyoakensis* did not close them.

As far as we know, this is the first report of rapid flower closure in response to mechanical stimulation of floral organs. In general, flower closure is induced by exogenous factors (e.g. light, humidity, pollination), and the following functions have been suggested to this habit. First, flower closure in response to rising humidity or declining temperature can protect reproductive organs from water and snow (van Doorn & van Meeteren 2003; van Doorn et al. 2014). However, this is not the case of the flower closure in *Drosera* spp. because we made experiments on sunny days. Second, plants often close a flower after pollination (Primack 1985) to reduce cost of flower maintenance (Ashman & Schoen 1994) or competition with unpollinated flowers of the same individual (He et al. 2005). This is also unlikely for the petal closure in *Drosera* spp. because the petal closure was not induced by stimulation of stamens or pistils where pollinators touch. Furthermore, the time required for flower closure in *Drosera* spp. was much faster (2-10 min) than typical flower closure after pollinator visit (1-2 hours to 2 days) (van Doorn 1997; He et al. 2005; Fründ et al. 2011). Third, nocturnal flower closure in many plant species functions as defense against florivory (Prokop & Fedor 2016). The petal closure of *Drosera* spp. may also function as defense against florivory because we observed caterpillars of sundew plume moth (*Buckleria paludum*) climbing the scape of *D. toyoakensis* in Toyoake (Tagawa & Watanabe, personal observation). *Buckleria paludum* is known as a specialist herbivore of *Drosera* spp., foraging on flowers (stamens, pistils and petals), fruits and glandular hairs on trap leaves (Eisner 2003; Matthews 2009). Whereas there is no quantitative study on floral damage by *B. paludum,* the fruits of *Drosera* are often damaged by *B. paludum* up to 40% in some habitats (Kataoka & Nishimoto 2007). Because *D. tokaiensis* can produce abundant seeds by selfing (Nakano et al. 2004), it may be adaptive for *D. tokaiensis* to close petals rapidly if it could detect a caterpillar of *B. paludum* climbing on a scape by any stimulation. To test this possibility, further studies on the response of *Drosera* spp. to the caterpillars of *B. paludum* is needed.

It is notable that the habit of petal closure to stimulation varies among *Drosera* spp. The pressure of florivory may differ among the species, and this expectation is to be tested in the future. While rapid plant movement has been studied since the time of Darwin (Darwin 1880), its variation among related species remain has been seldom documented (Simon et al. 2011). The discovery of rapid petal closure and its interspecific variation in *Drosera* spp. in this study has provided an extraordinary opportunity to study the physiological mechanisms and evolution of rapid plant movement.

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Figures and Tables

Figure 1 Time-lapse photographs of flower closure in response to mechanical stimulation on the calyx of *Drosera tokaiensis*, Flowers (a) before giving stimulation, (b) 2 min, (c) 3 min, (d) 5 min and (e) 3 h after giving stimulation are shown.

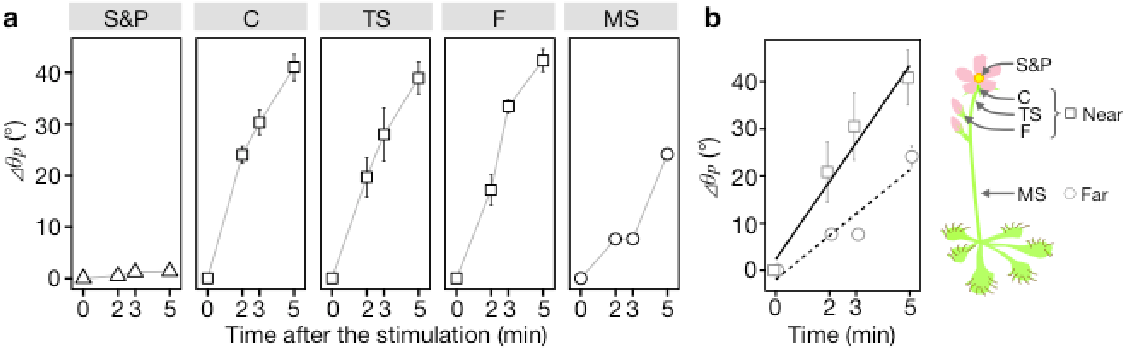

Figure 2 Change of petal openness $\angle\theta_{\mathrm{p}}$ before and 2, 3, 5 min after giving stimulation on plant organs. (a) Average changes of petal openness $\angle\theta_{\mathrm{p}}$ (± SE) giving stimulation on stamens and pistils (S&P, n = 4), calyx (C, n = 6), top of a scape (TS, n = 4), closed flower (F, n = 4) or middle position of a scape (MS, n = 3). (b) Average changes of petal openness $\angle\theta_{\mathrm{p}}$ (± SE) giving stimulation on the position near (C, TS or F) and far (MS) from a flower, and the expected line from the LMM (the solid line for the position near, and the dashed line for the position far from a flower).

Table 1 Results of the linear mixed model analysis to reveal factors affecting changes of petal openness ($\varDelta\theta_p$).

Explanatory variable	Estimate	SE	t	*P*
(Intercept)	-17.3	8.73	-1.97	0.07
Openness before stimulation	0.42	0.12	3.62	< 0.01
Time of stimulation	-5.94×10^{-4}	4.99×10^{-4}	-1.19	0.26
Time after the stimulation	4.64	1.14	-4.08	< 0.001
Position	1.04	4.45	0.23	0.82
Time after the stimulation × Position	3.59	1.26	2.86	< 0.01

Table 2 The proportion of flowers that closed 10 min after giving stimulation on stamens and pistils (S&P), calyx (C) or middle position of a scape (MS).

	S&P	C	MS
D. tokaiensis (July, Aichi)	0/4	6/6	3/3
D. spatulata (July, Miyazaki)	1/7	16/17	3/7
D. spatulata (July, Okinawa)	0/4	7/8	2/7
D. rotundiforia (July, Saga)	0/4	0/12	0/4
D. toyoakensis (July, Aichi)	0/4	0/4	0/4

Chapter 3. Hoverflies can sense the risk of being trapped by carnivorous plants: an empirical study using *Sphaerophoria menthastri* and *Drosera toyoakensis*

Abstract

Carnivorous plants are major predators of small insects in some habitats. Because traps of carnivorous plants are a serious threat for small insects, it is probable to evolve a mechanism to sense a cue of carnivorous plants and avoid being trapped. However, such a sensing behavior of small insects has never been described. Here we report that a hoverfly species *Sphaerophoria menthastri*, a major pollinator species of carnivorous sundew *Drosera toyoakensis*, exhibits a behavior to sense a cue of trap leaves and avoids landing there. In a quadrat (5 m × 5 m) where *D. toyoakensis* and other non-carnivorous plant species occur, we observed behaviors of hoverflies approaching *D. toyoakensis* and other plants. The numbers of approaches to trap leaves, flowers of *D. toyoakensis*, flowers of the other species and leaves of Poaceae and Cyperaceae were 9, 60, 52 and 54, respectively, and the numbers of landings to those four organs were 2, 55, 49 and 49, respectively. When *S. menthastri* approached trap leaves, they successfully avoided landing there by 1 or 2 hesitation behaviors, but were trapped unless it made a hesitation behavior. These findings suggest that *S. menthastri* can sense the trap leaves during an approach. Because hoverflies are major pollinators of *Drosera* spp., they may have effective avoidance mechanisms not for a particular species but for many species of *Drosera*.

Introduction

While predators of insects are usually animals, carnivorous plants are often major predators of small insects in some habitats. Because it is difficult for small insects to free themselves from trap leaves after being trapped (Gibson 1991; El-Sayed et al. 2016), any mechanism to avoid being trapped by carnivorous plants is considered to be advantageous for small insects living in habitats with carnivorous plants. Consequently, small insects trapped by carnivorous plants may have evolved a mechanism to sense a cue of carnivorous plants as is known in other prey animals that detect predators by visual (Amo et al. 2004), olfactory (Ninkovic et al. 2013) or vibrational (Remage-Healey et al. 2006) cues. Here, we test this idea by studying the relationship between a hoverfly *Sphaerophoria menthastri* (Syrphidae) and a carnivorous plant *Drosera toyoakensis* (Droseraceae).

Drosera toyoakensis has mucilaginous glands on trap leaves to catch and hold insects. Because *D. toyoakensis* has trap leaves close to flowers, insects visiting flowers including pollinator species are expected to be easily trapped. However, the frequency for a major pollinator species *S. menthastri* to be trapped by *D. toyoakensis* was quite low (Tagawa et al. 2018). This finding suggests that *S. menthastri* has a mechanism to avoid being trapped by *D. toyoakensis*. Previous studies on hoverflies suggested that hoverflies sense the existence of a predatory crab spider on a flower by “hesitation behavior” and avoid landing there (Yokoi & Fujisaki 2009; Suzuki & Yokoi 2016). “Hesitation behavior” is an action to hover forward and backward in front of a flower as if inspecting the safety of the landing site (Yokoi & Fujisaki 2009). We hypothesized that hoverflies could assess the existence of trap leaves by this hesitation behavior.

To test this idea, we set a quadrat in the habitat of *D. toyoakensis* and observed behaviors of hoverflies when approaching trap leaves and three other plant organs; flowers of *D. toyoakensis*, flowers of other species and leaves of other species. In this paper, we report that the results of this observation supported the above hypothesis.

Materials and Methods

Drosera toyoakensis (Droseraceae) is a carnivorous plant species with a single stem growing up to 10 to 20 cm. Linear and alternate trap leaves 5 to 7 cm long are scattered along the stem and trap leaves at the upper position are close to pink flowers. Trap leaves have glandular hairs and secrete sticky liquid and digestive enzyme (Adlassnig et al. 2010; Sun et al. 2014). *Sphaerophoria menthastri* (Syrphidae) is a hoverfly species 1.0 to 1.2 cm long. The species is a generalist pollinator and the most frequent flower visitor of *D. toyoakensis* in our study site (Tagawa et al. 2018). We made field observations from 8 am to 2 pm on July 16-17, August 18, and September 2-3, 2014, in a quadrat (5 m × 5 m) located in a protected bog at Toyoake (N 35°4'26" E 137°1'7", Toyoake City, Aichi Pref., Japan). Under the permission from Toyoake City, we observed and counted the number of approaches of *S. menthastri* to four plant organs: flowers of *D. toyoakensis*, trap leaves of *D. toyoakensis*, flowers of a co-occurring non-carnivorous plant species *Lysimachia fortunei* and leaves of some species of Poaceae and Cyperaceae. We confirmed that there were no predators (e.g. crab spiders and praying mantis) existed on flowers and leaves in the quadrat. *Sphaerophoria menthastri* did not approach and land on leaves of *L. fortunei* that were small (ca. 1-2 cm long) and mostly covered by dense leaf layers of Poaceae and Cyperaceae. When a hoverfly approached an organ, we counted the number of hesitation behaviors by defining a hesitation behavior as a sequence of one forward and one backward flight in front of an organ (Yokoi & Fujisaki 2009). We also recorded whether or not a hoverfly landed on an organ after a series of hesitation behaviors. We finished recording the behavior of an individual of *S. menthastri* when it flew away from the survey plot. We defined landing percentage for each organ as the number of landings divided by the number of approaches. To test a difference in the number of approaches among four types of plant organs, we made a generalized linear mixed model (GLMM) with the number of approaches as a response variable, the four types of organ as explanatory variables and individual ID as a random factor. The error distribution is Poisson with log link function. To test a difference in the number of hesitation behaviors among organs to which hoverflies approached, we used another GLMM with the number of hesitation behaviors as a response variable, the four types of plant organs as an explanatory variable, and individual ID as a random factor. The error distribution is Poisson with log link function. Furthermore, another GLMM was applied to test whether there was a significant difference in the landing percentage among plant organs to which hoverflies approached. Bonferroni-Holm adjustments of type I error for multiple comparisons were used in the above three tests. All statistical analyses were made using R 3.1.3 (R Core Team 2010) with a package “lme4” (Douglas et al. 2015).

Results

The observed numbers of approaches to trap leaves, flowers of *D. toyoakensis*, flowers of the other species and leaves of Poaceae and Cyperaceae were 9, 60, 52 and 54 respectively. The number of approaches of hoverflies was significantly lower for trap leaves than flowers of *D. toyoakensis*, other flowers, and other leaves (Fig. 2, GLMM $P < 0.05$). The observed numbers of landings to trap leaves, flowers of *D. toyoakensis*, other flowers and other leaves were 2, 55, 49 and 49, respectively. The landing percentage for trap leaves (22.2%) was significantly lower than for the other plant organs (91.7% for flowers of *D. toyoakensis*, 94.2% for other flowers, 90.7% for other leaves; Fig. 3b, GLMM $P < 0.05$). The number of hesitation behaviors to trap leaves (mean ± SE: 1.00 ± 0.236, N = 9) was not significantly different from the numbers of hesitation behaviors to other organs including flowers of *D. toyoakensis* (1.40 ± 0.197, N = 60; Fig. 3a, GLMM $P < 0.05$). On the other hand, the number of hesitation behaviors to other leaves (0.556 ± 0.162, N = 54) was significantly fewer than that to other flowers (1.60 ± 0.244, N = 52; GLMM $P < 0.05$). Among nine individuals observed, seven exhibited one or two hesitation behaviors in front of trap leaves and avoided landing there. The rest two individuals did not exhibit any hesitation behavior and were trapped by leaves.

Discussion

A hover fly *S. menthastri* approached trap leaves less frequently than three other plant organs: flowers of *D. toyoakensis*, flowers of the other species and leaves of Poaceae and Cyperaceae. In addition, it landed less frequently on trap leaves than on three other plant organs. When *S. menthastri* approached trap leaves, *S. menthastri* successfully avoided landing there by 1 or 2 hesitation behaviors, but *S. menthastri* was trapped unless it made a hesitation behavior. These findings suggest that *S. menthastri* can sense the existence of trap leaves before approaching and also during hesitation behaviors after an approach.

A hoverfly *S. menthastri* is likely to use visual and/or chemical cues to sense the existence of trap leaves. Although there has been no quantitative study, UV-sensitive photograph shows that viscous liquid secreted from glandular hairs on traps of carnivorous *Drosera* spp. reflects UV and make a visual contrast against the background leaves absorbing UV (Joel et al. 1985). *Sphaerophoria menthastri* can sense this visual contrast because hoverflies have tetrachromatic color vision with sensitivity from UV, blue, green to yellow wavelength (Kelber 2001; Woodcock et al. 2014). *Sphaerophoria menthastri* may also use chemical cues to sense trap leaves because trap leaves of *D. toyoakensis* emit strong sweet odor (Tagawa pers. obs.). Experiments with an artificial model baited with trap odor as in a previous study (El-Sayed et al. 2016) are needed to identify chemical cues that *S. menthastri* uses.

The number of hesitation behaviors was 1 or 2 when *S. menthastri* approached trap leaves, showing that *S. menthastri* sense trap leaves quickly. On the other hand, when approaching flowers, *S. menthastri* exhibited 2 or more hesitation behaviors in nearly half of the approaches (46.3%). This difference may reflect the difference in constancy of predation risks in each place to approach; risks of predation in flowers are occasional while the risk of predation by trap leaves is constant. In this case, it is advantageous for *S. menthastri* to sense trap leaves with as few hesitation behaviors as possible and fly away from the place. Hoverflies are reported as major pollinator species in other species of *Drosera* (Murza & Davis 2005; El-Sayed et al. 2016), and thus hoverflies may have evolved effective avoidance mechanisms not for a particular species but for many species of *Drosera*. Further studies on hoverflies and other sundew species are needed to deepen our understanding on carnivorous plants-prey interactions.

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Figures and Tables

Fig. 1 A hoverfly species *Sphaerophoria menthastri* visiting a flower of *Drosera toyoakensis*.

Fig. 2 The mean number (± SE) of approaches of a hoverfly species *Sphaerophoria menthastri* to four types of plant organs (observed individuals: n = 57).

Letters above the bars indicate significant differences at 5% level (GLMM with Bonferroni-Holm adjustments).

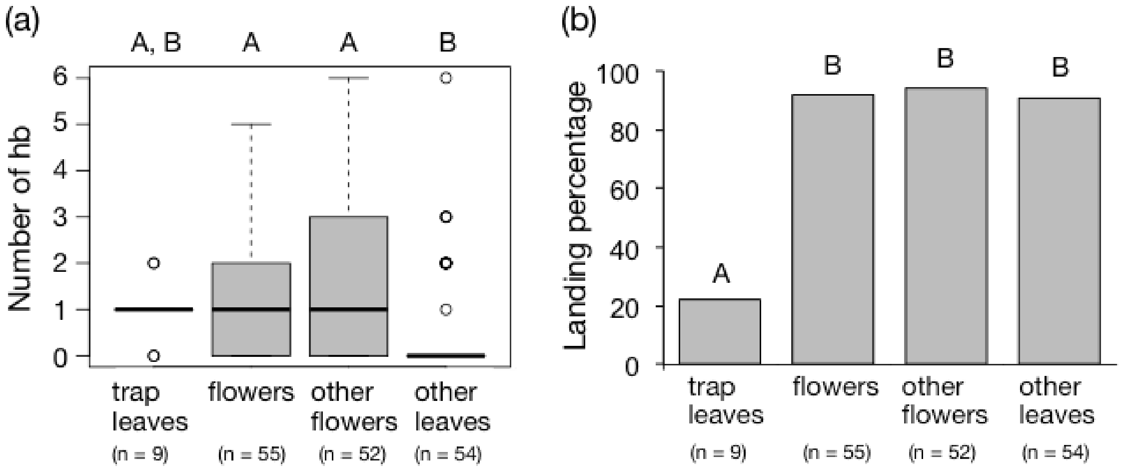

Fig. 3 The number of hesitation behaviors (hb) and landing percentage by a hoverfly species *Sphaerophoria menthastri* to four types of plant organs.

(a) The number of hesitation behaviors and (b) the landing percentage when approaching four types of plant organs (trap leaves of *Drosera toyoakensis*, flowers of *D. toyoakensis*, flowers of a non-carnivorous plant species *Lysimachia fortunei* and leaves of Poaceae and Cyperaceae). Letters above the bars indicate significant differences at 5% level (GLMM with Bonferroni-Holm adjustments).

Chapter 4. Co-occurring neighbor plants reduce the trapping efficiency of a carnivorous plant, *Drosera rotundifolia* (Droseraceae)

Abstract

Carnivorous plants usually grow in open habitats, and it has been considered that the dense vegetation of co-occurring plants affects the fitness of carnivorous plants negatively. However, there has been no quantitative research to test the effect of them *in situ*. We showed here for a rosette-leaf sticky carnivorous plant species *Drosera rotundifolia* (Droseraceae), co-occurring plants affected negatively the trapping efficiency directly (decreasing the visibility) and/or indirectly (decreasing the mucilage hairs on trap leaf to retain prey). There was a negative effect of the biomass of co-occurring plants on the number of mucilage hairs and the trapped prey individuals of *D. rotundifolia*. The number of prey individuals belonging to Diptera decreased with the increase of the biomass of the co-occurring plants and the rate of trap leaves covered by co-occurring plants (cover rate). It may be because the visibility and attractiveness decreased with the increase of the cover rate. Our research suggests that cutting co-occurring vegetation regularly and keeping the habitat open is important for the conservation of the rosette-form carnivorous plants species like *D. rotundifolia.*

Introduction

Carnivorous plants, which catch arthropods by specialized traps and absorb nutrients from them (Darwin 1875), had evolved at least 10 times independently and now about >800 species are known around the world (Fleischmann et al. 2018). However, the habitats of carnivorous plants are limited, because the environment that the benefits outweigh the costs of carnivory is limited. A principal factor determining the benefits of carnivory is the quantity of prey and the efficiency of prey use (Ellison and Adamec 2018). In fact, the increase of the amount of prey leads to the increase of the photosynthetic rate, the number of flowers and seeds (Ellison 2006; Pavlovič and Saganová 2015). Therefore, it is important to know what environmental factors affect the quantity of prey for understanding the habitat limitation of carnivorous plants.

The quantity of prey is affected by both abiotic and biotic environmental factors. Abiotic environmental factors (e.g. temperature, humidity, solar radiation) affect the quantity of tapped prey as is known in carnivorous *Pinguicula moranensis* that the prey capture increased towards the shadiest, most humid, and fertile population parallel to the prey availability (Alcalá and Domínguez 2003). Biotic environmental factors such as animals and plants that cohabit with carnivorous plants also affect the quantity of prey. Some spiders and a toad species compete for arthropod prey with carnivorous *Drosera capillaris* and decrease the quantity of trapped prey (Jennings et al. 2010, 2016). Co-occurring plants may affect the quantity of trapped prey positively and negatively. When co-occurring plants have attractive flowers, carnivorous plant species exploit and trap their pollinator species effectively and increase the quantity of prey as is known in carnivorous *D. makinoi* and *D. toyoakensis* (Tagawa et al. unpublished). On the other hand, co-occurring plants are likely to affect the quantity of prey negatively by two non-exclusive mechanisms. First, because large co-occurring plants make the light-limited environment for small carnivorous plants, they decrease the investment for the carnivory plastically, which result in trapping smaller amount of prey. In the light-limited environment, the photosynthetic rate of carnivorous plants is limited not by nutrient gain but by light, and carnivorous plants usually decrease the resource investment for trapping organs (Zamora et al. 1998; Guisande et al. 2004; Alcalá and Domínguez 2005). The decrease of the investment for carnivory may lead to the decrease of the amount of prey. Second, dense vegetation around an individual of carnivorous plant may decrease the visibility by hiding traps from above (Gibson 1983), and make it difficult for flying insects like Diptera, the main prey family of *Drosera* spp. (Darnowski et al. 2018) to approach traps. Although usually carnivorous plants cohabit with non-carnivorous plants and it is predicted that non-carnivorous plants affect the trapping efficiency as mentioned above, there has been no quantitative report about the effect.

In this study, we quantified the characteristics of co-occurring vegetation (cover, height, covered proportion of trap leaves), the traits of traps (number of mucilage hairs, leaf area, height) and the amount of prey for carnivorous *D. rotundifolia* occurring in a habitat that has an environmental gradient in the density of the co-occurring vegetation. We hypothesized two causal relationships between three types of parameters. First, co-occurring vegetation decreases the amount of prey through changing the trait of traps (e.g. decreasing the investment for a trapping organ: mucilage hairs). Second, co-occurring vegetation decreases the amount of prey directly by decreasing the easiness of approaches and the visibility of traps. We verified the two hypotheses by conducting path analyses connecting three types of parameters.

Materials and Methods

Material and study site

Drosera rotundifolia (Droseraceae) is a perennial carnivorous plant species, which forms rosette with ladle-shaped leaves (Kagawa 2015). Arthropods are captured by the sticky mucus produced by mucilage hairs on the upper surface of the leaf (Thoren et al. 2003). Adding prey to trap leaves increases the growth rate and the reproductive success of *D. rotundifolia* (Krafft and Handel 1991). We made a field survey in Mt. Tenzan in Karatsu City, Saga Prefecture, Japan (N 33°20’21” E 130°8’35”) on September 23rd 2016. In this mountain, *D. rotundifolia* grows widely in half-open places along a trail, neighboring to dense vegetation of *Sasa nipponica* (Poaceae). We selected 20 individuals of *D. rotundifolia* for the experiment randomly.

Quantification of the trait of traps and the trapping efficiency

For each individual of *D. rotundifolia*, we quantified three parameters to indicate the traits of traps: number of mucilage hairs, leaf area and the covered proportion of *D. rotundifolia* traps. We measured and noted the height in the field. We took photographs of two trap leaves for an individual in the experiment room with a digital camera (Olympus tough TG-3, Tokyo, Japan), and counted the number of mucilage hairs and measured the trap leaf area using the photographs with an image processing software Image J (Schneider et al. 2012). We collected and counted all prey individuals trapped by each *D. rotundifolia* with a pair of tweezers and identified them in the family level.

Quantification of the trait of the co-occurring vegetation

For each individual of *D. rotundifolia*, we quantified three parameters to indicate the trait of the co-occurring vegetation: cover of co-occurring vegetation, the height of co-occurring plants and the covered proportion of *D. rotundifolia* traps. We randomly selected and measured the height of three non-carnivorous plant individuals within a 20 cm circle around a focal *D. rotundifolia* individual, and averaged them to use as the height of co-occurring plants. In order to measure the cover of co-occurring vegetation, we took photographs of each *D. rotundifolia* individual with surrounding vegetation using a digital camera (Olympus tough TG-3, Tokyo, Japan) at a height of 20 cm. Converting these photographs to an image processing software Image J (Schneider et al. 2012), we counted the number of pixels with green leaves and stems of surrounding vegetation manually (Supplemental Figure 1). Then we divided the number of pixels of surrounding vegetation by the number of all pixels to get cover of the co-occurring vegetation. We calculated the covered proportion of *D. rotundifolia* traps to divide the number of traps hidden by surrounding vegetation from above by the number of all traps.

Statistical Analyses

In order to determine whether there was a significant effect of co-occurring vegetation on trapping efficiency, we made single regressions with generalized linear models (GLMs). The response variable was the number of prey individuals, the number of individuals of trapped Diptera (main family of prey in *D. rotundifolia*), the binary data whether prey was trapped or not, or the binary data whether Diptera was trapped or not, and the explanatory variable was the height of co-occurring plants, cover of co-occurring vegetation or the covered proportion of *D. rotundifolia* traps. When the response variable was the number of individuals of prey or Diptera, we used a negative-binomial error distribution and a log link function (Zuur et al. 2009). When the response variable was binary data of prey or Diptera, we used a binomial error distribution and a logit link function. Next, we conducted path analyses using parameters of co-occurring vegetation that showed the significant effects in the previous GLM analyses: the height of co-occurring plants and the cover of co-occurring plants. We hypothesized a causal relationships; the co-occurring vegetation decreases the trapping efficiency through changing the feature of traps (Figure 1). We computed all statistical analyses using R 3.1 (R Core Team 2010) with packages “MASS” (Venables and Ripley 2002) and “piecewiseSEM” (Lefcheck 2016).

Results

The cover of co-occurring vegetation affected the number of trapped insects, the number of trapped Diptera and the probability of trapping Diptera negatively (Table 1, Figure 2 a-c). The height of co-occurring plants affected the probability that Diptera was trapped negatively (Table 1, Figure 2 d). The covered proportion of trap leaves did not affect any parameters of the trapping efficiency significantly.

Path analyses showed that the cover of co-occurring vegetation affected the number of mucilage hairs negatively (Figure 3), while the three types of parameters of features of traps including number of mucilage hairs did not affect the three types of parameters of trapping efficiency (The number of prey, the number of trapped Diptera and the probability of trapping Diptera) significantly. Therefore, the causal hypothesis that the co-occurring vegetation decreases the trapping efficiency through affecting negatively the feature of traps was not supported.

Discussions

The increase of cover of co-occurring vegetation significantly decreased the number of trapped insects, trapped Diptera and the probability of trapping Diptera. The height of co-occurring plants affected the probability of trapping Diptera negatively. The cover of co-occurring vegetation affected the number of mucilage hairs negatively. Neither the number of mucilage hairs, leaf area nor the height of traps affected the quantity of prey significantly.

The decrease of the number of mucilage hairs with the increase of cover of co-occurring vegetation may be due to the limitation of light and the decrease of the investment for carnivory as was shown in other carnivorous plant species: the decrease of the number of bladders in *Utricularia* and the density of glands in *Pinguicula* with the decrease of light level (Zamora et al. 1998; Guisande et al. 2004). However, the decrease of the number of mucilage hairs did not affect the quantity of prey significantly. Therefore, the decrease of the quantity of prey parallel to the increase of the co-occurring vegetation was induced not by the changes of features of traps (the number of mucilage hairs, trap area and height of traps) but by other factors. The height of co-occurring plants did not affect the number of mucilage hairs, but affected the probability of trapping Diptera negatively. So, the height of co-occurring plants, too, affected the quantity of prey without changes of the feature of traps. It is likely that the dense vegetation with large cover and tall height prevents insects including Diptera from flying smoothly and approaching traps of *D. rotundifolia*. Some experiments setting artificial adhesive models on places differing in the cover and height of surrounding vegetation will help to verify the hypothesis.

Recent studies suggested that many of *Drosera* spp. trap insects accidentally like webs of spiders rather attracting insects (Foot et al. 2014; Potts and Krupa 2016). In this case, the surrounding environment may maintain the quantity of prey, which limits the fitness of carnivorous plants. Our study showed that the co-occurring plants are likely to change the structure of the environment around *D. rotundifolia* and the quantity of prey.

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Figures and Tables

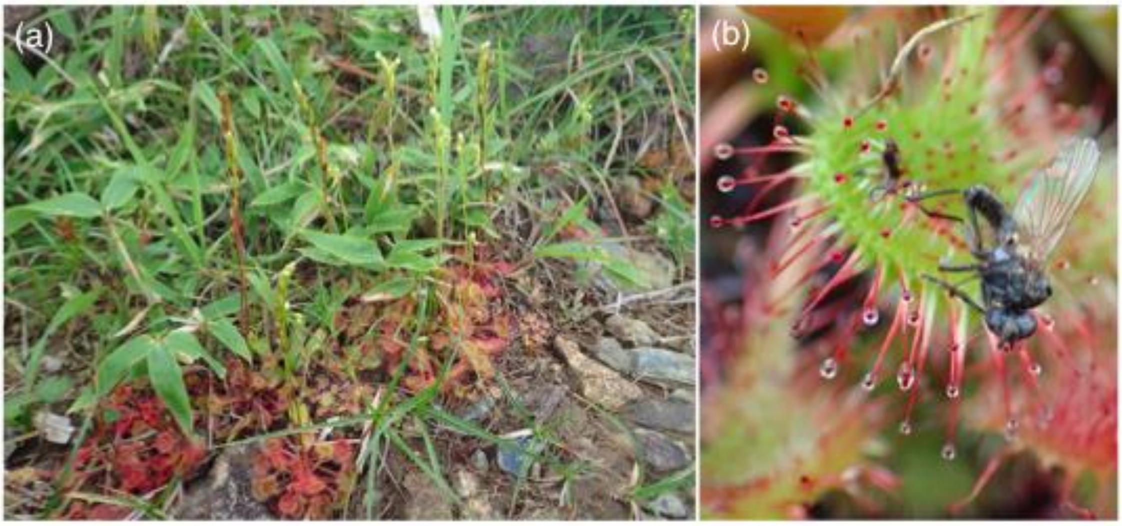

Figure 1. Carnivorous *Drosera rotundifolia* and its habitat. (a) *Drosera rotundifolia* grows in half-open places along the trail, neighboring to the dense vegetation of *Sasa nipponica*. (b) *Drosera rotundifolia* mainly traps insects belonging to Diptera.

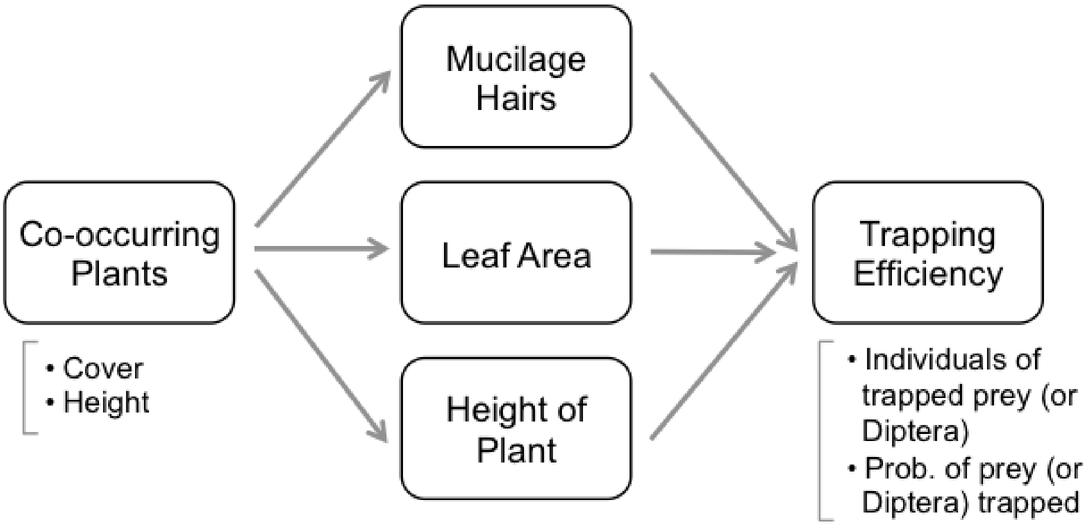

Figure 1. A path analysis diagram to show the hypothesis that co-occurring plants affect the trapping efficiency of *Drosera rotundifolia* through changing the traits of traps.

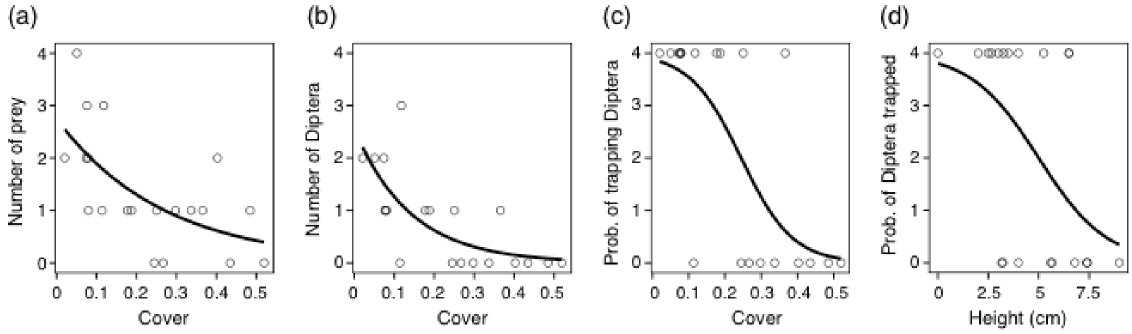

Figure 2. The co-occurring vegetation affected the trapping efficiency negatively. The cover of co-occurring vegetation affected negatively (a) the number of all prey, (b) the number of trapped Diptera and (c) the probability of trapping Diptera. (d) The height of co-occurring plants affected the probability of trapping Diptera negatively.

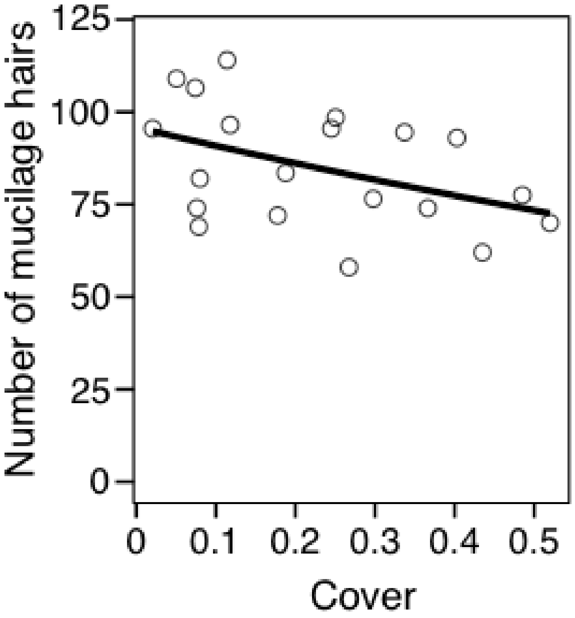

Figure 3. The cover of co-occurring vegetation negatively affected the number of multiage hairs.

Table 1. Effects of co-occurring vegetation on the trapping efficiency of *Drosera rotundifolia.*

Explanatory variable	Coefficient	Statistic	*P* value
(I) Response variable: Individuals of trapped insects			
Cover	-3.73 ± 1.55	-2.41	**0.01**
Height	-0.095 ± 0.089	-1.07	0.28
Prop. of traps covered	-1.16 ± 0.657	-1.76	0.07
(II) Response variable: Probability of insects trapped			
Cover	-8.41 ± 4.69	-1.79	0.07
Height	-0.911 ± 0.47	-1.91	0.06
Prop. of traps covered	-1.75 ± 1.51	-1.16	0.25
(III) Response variable: Individuals of trapped Diptera			
Cover	-7.07 ± 2.61	-2.71	**0.006**
Height	-0.146 ± 0.117	-1.25	0.21
Prop. of traps covered	-1.99 ± 1.04	-1.91	0.06
(IV) Response variable: Probability of Diptera trapped			
Cover	-14.2 ± 5.83	-2.43	**0.01**
Height	-0.591 ± 0.288	-2.05	**0.04**
Prop. of traps covered	-2.71 ± 1.49	-1.82	0.06