

Functional significance of petals as landing sites in fungus-gnat pollinated flowers of *Mitella pauciflora* (Saxifragaceae)

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Summary

1. Despite the well-known visual attraction function of angiosperm petals, additional roles of these floral organs (e.g. the provision of landing-site platforms for pollinators) have rarely been examined. This is likely because most petals perform multiple functions, making it difficult to isolate the importance of landing sites in pollination success.

2. We investigated the landing-site function of dull-coloured pinnately branched petals in *Mitella pauciflora* flowers, which are predominantly pollinated by fungus gnats. We conducted a field experiment, in which the effects of experimental petal removal on pollinators' approach, landing and visit duration and floral reproductive success were examined in naturally pollinated flowers.

3. According to direct and time-lapse camera observations, petal removal did not influence pollinators' approach frequency or visit duration, but did significantly decrease their landings. Fruit set and pollen dispatch both significantly decreased with petal removal, indicating that petals promote female and male reproductive success in *M. pauciflora* by facilitating pollinator landing.

4. This demonstrates that inconspicuous petals primarily have a landing-site function rather than a visual attraction function in *M. pauciflora*. Discriminating between diverse petal functions is a challenging problem, and new approaches are required to elucidate the functional features of angiosperm flowers.

Key-words: fungus gnat, landing-site function, *Mitella*, pollination success, pollinator approach, visual attraction function

Introduction

The amazing diversity of flowers in angiosperms can be demonstrated by the high degree of inter and/or intraspecific variation in the number, size, shape, colour and spatiotemporal arrangement of basic floral organs such as petals, sepals, pistil and stamens (Harder & Barrett 2006). The diversity of floral organs is thought to have evolved to maximise pollen transfer by adaptation to specific morphologies and behaviours of respective pollinators (e.g. Nilsson 1988; Johnson & Steiner 2000; Fenster *et al.* 2004; Wilson *et al.* 2004).

Petals, which are commonly situated in the second whorl of a differentiated perianth, are extremely diverse in terms

of morphology within and among taxonomic groups of flowering plants (Irish 2009; Rasmussen, Kramer & Zimmer 2009; Ronse De Craene & Brockington 2013). Although petal diversity has been thought to evolve in relation to the diverse visual attraction functions of pollinators, it is difficult to explain all floral diversity solely in relation to this function (Endress 1994). Petals store nectar (Kramer & Hodges 2010), apply pollen to pollinators (Tomlinson, Primack & Bunt 1979; Almeida *et al.* 2013), emit olfactory attractants (Dötterl & Jürgen 2005; Balao *et al.* 2011) and act as a landing foothold or platform for pollinators (Kampny 1995; Giovanetti & Aronne 2013). For example morphologically specialised petals such as the lips in Orchidaceae (Benítez-Vieyra *et al.* 2006) and keel petals in Fabaceae (Le Roux & Van Wyk 2012) often serve as landing platforms to specialist pollinators. Furthermore, recent studies have revealed that most flowers have conical

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(papillate) epidermal cells, which facilitate pollinators gripping the flowers on petal surfaces (Kevan & Lane 1985; Whitney *et al.* 2009; Alcon, Whitney & Glover 2012).

Because most petals have multiple roles, it is not easy to isolate the importance of the landing-site function in pollination success (e.g. Temeles & Rankin 2000; Zung *et al.* 2015). Using mutants lacking conical epidermal cells, Whitney *et al.* (2011) investigated the petal landing-site function, and found that the cells had numerous functions in addition to facilitating pollinator landings. Castellanos, Wilson & Thomson (2004) found that petals could control visit duration and/or pollinator movements within a flower after landing, but there has been little other research related to this.

In this study, we investigated the landing-site function of petals in the scent-attracting flowers of *Mitella pauciflora* (Saxifragaceae), which are predominantly pollinated by fungus gnats (Okuyama, Pellmyr & Kato 2008). Fungus-gnat pollinated flowers are ideal subjects with which to test the landing-site function, because their petals are likely to be less involved in visual attraction than those of bee- or syrphid fly-pollinated flowers. Fungus-gnat pollination is found in many forest-floor herbs, such as *Aristolochia*, *Heterotropa* (Aristolochiaceae), *Arisaema* (Araceae), *Scolopus* (Liliaceae), *Tolmiea* (Saxifragaceae), *Corybas*, *Lenpanthus* and *Pterostylis* (Orchidaceae), which generally have dull-coloured (greenish, and/or pale or dark red) tepals, and/or floral scents (Mesler, Ackerman & Lu 1980; Sugawara 1988; Goldblatt *et al.* 2004; Blanco & Barboza 2005; Barriault *et al.* 2010). *Mitella* species have inconspicuous and pinnately branched petals, which are typical floral features of fungus-gnat pollinated flowers found in dark forest-floor environments (Mesler, Ackerman & Lu 1980; Goldblatt *et al.* 2004; Okuyama, Kato & Murakami 2004; Woodcock *et al.* 2014). Based on behavioural observations in the field, the inconspicuous petals in *Mitella* flowers have been hypothesised to act mainly as a foothold or landing platform for pollinators (Fig. 1; Okuyama, Kato & Murakami 2004; Endress & Matthews 2006), but no direct empirical tests have been conducted.

Here, we conducted a petal-removal experiment that examined the effects of pinnately branched petals on the number of approaches and landings, visit duration, successive probes per visit by pollinators and the reproductive success of naturally pollinated *M. pauciflora* flowers. We postulated that petal removal would decrease (i) the frequency of landing after pollinator approach to the flowers, and/or (ii) the duration of pollinator visits, but would have little effect on pollinator approach frequency.

Materials and methods

STUDY SPECIES AND SITE

Mitella pauciflora is a Japanese endemic perennial herb species that usually grows on moist forest floors and along forest streams. The inflorescences produce 2–10 perfect flowers, with five pinnately branched, pale green or very pale red petals that grow up to 20–30 cm in length: large inflorescences usually have more flowers than smaller inflorescences. Flowers within an inflorescence usually open simultaneously and secrete nectar as a floral reward. The species attracts a specific pollinator group, short-tongued fungus gnats that are 5–6 mm in body size (Fig. 1), using floral scents such as linalool and β -caryophyllene, and a nectar reward (Okuyama, Pellmyr & Kato 2008; Okuyama 2012; Okamoto *et al.* 2015). Okuyama (2016) reported that in *M. pauciflora* most linalool was emitted from floral organs other than petals; β -caryophyllene was released by a range of floral organs, including the petals. He detected intra-population variation in β -caryophyllene emissions in this species. No β -caryophyllene was released from the flowers of some individuals. Petal removal had relatively little effect on floral scent composition (Okuyama 2016). According to our preliminary hand-pollination experiment, *M. pauciflora* is weakly self-compatible but not autonomously self-pollinated, so the species requires pollinators for reproduction (Fig. S1, Supporting Information).

We conducted the study in the Ishikawa Forest Experiment Station, Ishikawa, Japan (36°25'45" N, 136°38'34" E) during the flowering period from April 2015 to early May 2015. We examined six flowering patches within the study site (c. 30–100 m²), which were separated by roads.

PETAL REMOVAL EXPERIMENTS IN THE FIELD

To examine the functions of pinnately branched petals, we arbitrarily selected unvisited newly opened inflorescences and prepared three experimental inflorescence types: unmanipulated control (C),



Fig. 1. Fungus gnat (left) and crane fly (right) that visited flowers of *Mitella pauciflora*. These insects grasped pinnately branched petals during landing.

in which all petals within inflorescences were kept intact; partial petal removal (PR), in which the tip of each petal was removed from all flowers within inflorescences; and complete petal removal (R), in which all petals were completely removed (Fig. 2). Petal removal was conducted with tweezers or dissection scissors. We conducted the PR treatment to examine the effect of removal damage on pollinator behaviour, as damaged parts can emit repellent volatiles (Howe & Jander 2008). For the purposes of our experiment, we arbitrarily chose neighbouring inflorescences separated by distances that varied according to inflorescence density; the minimum distances between nearest neighbouring inflorescences at our study site (in 2016) were c. 4–30 cm. The numbers of inflorescences examined for each inflorescence type are shown in the following sections.

POLLINATOR OBSERVATION

To examine whether petal removal decreases pollinator visits over long and short time periods, we conducted two types of observations: time-lapse camera and direct observations.

Time-lapse camera photography

Using time-lapse cameras, we observed pollinator landings on experimental inflorescences from 9–17 April. In front of each experimental inflorescence, we set a single time-lapse camera (PENTAX WG-1, PENTAX WG-3 or RICOH WG-20) to take pictures at 120- or 30-s intervals. The cameras set at 120-s intervals took pictures for approximately 24 h, whereas cameras set at 30-s intervals took pictures for approximately 7 h. The observation period varied slightly depending on camera conditions. Because fungus gnats visit *Mitella* flowers most frequently in the evening (Okuyama, Kato & Murakami 2004), we started each photography session after 16.00 h. In total, we examined 111 inflorescences with time-lapse cameras (120-s interval observation: R = 14, PR = 8 and C = 28; 30-s interval observation: R = 20, PR = 14 and C = 27). We visually inspected photographs on a PC monitor to identify pollinator type (fungus gnat, crane fly or others; Fig. S2), counted the number of landings by each pollinator type on each experimental inflorescence during the photography period, and then calculated the number of landings per hour for each experimental inflorescence. We also examined the visit duration per flower, and the number of successive probes per visit of each pollinator individual by counting the number of photographs in which each individual was recorded continuously on a given flower, and by checking pollinator movements on an examined inflorescence, respectively.

Direct observation

Because photography was not suitable to investigate pollinator approach behaviour, we conducted direct pollinator observations for 2 days in a single patch. We arbitrarily selected 78 inflorescences, counted flower numbers per inflorescence, and prepared control (C = 41) and complete petal removal (R = 37) inflorescences. We then observed pollinator approaches and landings on each experimental inflorescence from 16.30 to 18.00 h. While approach is often described as a pollinator finding and drawing near to a flower from the front (e.g. Ushimaru & Hyodo 2005; Ushimaru *et al.* 2009), in this study, we defined an approach as a pollinator touching any part of a flower or inflorescence with or without subsequent landing, because application of the first definition to fungus gnats is difficult due to their quick and unsteady fluttering. Landing was defined as a pollinator landing on a flower and foraging nectar within a given inflorescence. We recorded the visit duration (s) as the time between a pollinator landing on a

flower and flying away. Flower visitors were categorised into three groups: fungus gnats, crane flies and others.

REPRODUCTIVE SUCCESS

Approximately 3 weeks after anthesis, on 30 April–1 May, we assessed fruit and seed production as a measure of female reproductive success. We examined fruit set (fruit number/flower number) in five patches of naturally pollinated inflorescences (R = 45, C = 39). We collected mature fruits and counted the number of seeds per fruit. In total, we examined 51 and 56 fruits from R and C inflorescences respectively.

In addition, we estimated male reproductive success by counting the number of pollen grains remaining on the anthers (c.f. Ushimaru, Kawase & Imamura 2006; Ushimaru *et al.* 2009; Ushimaru, Kobayashi & Dohzono 2014). We collected middle-positioned flowers within inflorescences (R = 27, C = 27) at both 3 and 6 days after flower opening. We also sampled a newly opened, unvisited middle-positioned flower from each of 27 inflorescences to examine pollen production per flower (135 flowers in total). These flowers were stored in 1.0 mL of 70% ethanol and mashed with a stick to detach pollen grains from anthers in the solution. We estimated the number of remaining pollen grains per flower by counting the pollen numbers in three 10.0 μ L drops per sample under a microscope.

DATA ANALYSES

Pollinator behaviour

Using time-lapse camera data, we tested the effects of petal removal on (i) the number of pollinator landings per inflorescence and (ii) successive probes per visit. We constructed a generalised linear mixed model (GLMM) with a negative binomial error (logarithmic link) in which inflorescence type (R, PR or C), display size (i.e. the number of flowers per inflorescence) and time interval type (30 or 120 s) were incorporated as the explanatory variables, the number of pollinator landings was the response variable. Because display size can also influence pollinator attraction in fungus-gnat pollinated plants, we included display size as a covariate in this and subsequent analyses. We also incorporated the

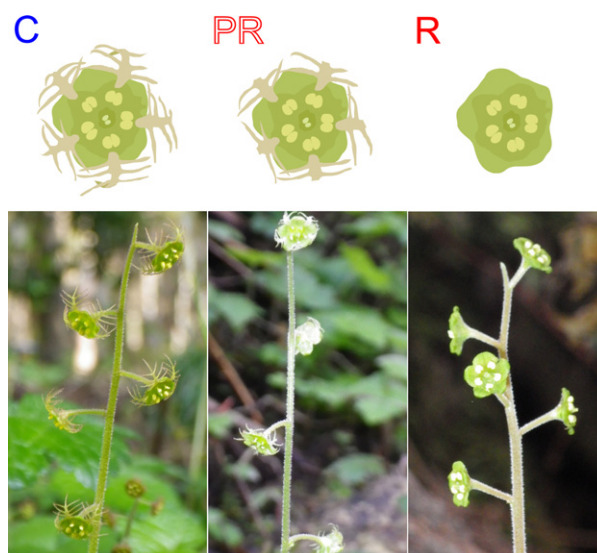


Fig. 2. Experimental inflorescence types: C, all petals were kept intact; PR, the tips of each petal were removed in all flowers; R, all petals were completely removed.

duration of observation (h) as an offset term in the model; this procedure allowed us to analyse landing frequency (number of landings per hour). The observation date and patch identities were incorporated as random terms. In addition, we constructed a GLMM with a negative binomial error (logarithmic link) that incorporated inflorescence type, display size and time-interval type as the explanatory variables, the total number of flowers visited per inflorescence as the response variable, the total number of pollinator individuals visiting the inflorescence as an offset term, and the observation date and patch identities as random terms.

We tested petal function in pollinator attraction by comparing approach and landing numbers between R and C inflorescences using the direct observation dataset. We constructed a GLMM with negative binomial error (logarithmic link) in which inflorescence type (R or C) and display size were included as the explanatory variables, the number of approaches was the response variable and the observation date identity was a random term. We also constructed GLMM with binomial error (logit link) in which types of petal removal and display size were the explanatory variables, the landing to approach ratio was the response variable and the observation date identity was a random term.

We tested the effect of petal removal on visit duration per flower using a GLMM (Gamma error and logit link). In the model, inflorescence type (R or C) was the explanatory variable and the observation date, and inflorescence and pollinator identities were random terms. The number of photographs in which each individual was recorded continuously on a given flower, and visit duration (s), were the response variables for the time-lapse camera and direct observation date sets respectively. The 30 or 120-s datasets were analysed separately.

Female and male success

We examined the effects of petal removal on fruit and seed set and pollen transfer using GLMMs. We constructed a GLMM with binomial error (logit link) in which inflorescence type (R or C) and display size were included as the explanatory variables, fruit set per inflorescence was the response variable and patch identity was a random term. We used a GLMM with a negative binomial error (logarithmic link) to test the effect of seed set; inflorescence type (R or C) and display size were explanatory variables, and the number of seeds per fruit and the patch identity were the response variable and a random term respectively. In addition, we constructed a GLMM with a negative binomial error (logarithmic link) in which inflorescence type, display size and days after opening were incorporated as the explanatory variables, the number of remaining pollen grains per flower was the response variable and patch identity was a random term.

We examined the significance of each explanatory variable in the GLMMs using a Wald test. We performed all analyses using the *glmmADMB* package in R software (ver. 3.1.1; R Development Core Team, 2010).

Results

POLLINATOR BEHAVIOUR

We recorded landings by 255 pollinator individuals with time-lapse cameras (120-s interval, $n = 187$; 30-s interval, $n = 68$) and 63 pollinators by direct observation (Fig. S2). Fungus gnats accounted for approximately 80% (199 individuals) of the total pollinator individuals in time-lapse camera observation. Landings by crane flies and others each accounted for approximately 10% of total pollinator individuals (29 and 27 individuals respectively). In direct

observation, we found only fungus-gnat visits to experimental inflorescences. We did not observe specific fungus-gnat assessment behaviours (hovering with antennal contact to the floral organs) prior to landing on the flowers; these behaviours have been reported in species of bees and syrphid flies (c.f. Lunau 2000; Ushimaru, Watanabe & Nakata 2007). Most of the fungus gnats landed on flowers immediately after approach (Fig. 1). Fungus gnats rarely landed on the inflorescence stems first, moving thereafter to a nearby flower.

According to the GLMM analysis for time-lapse camera data, R treatment significantly decreased the number of pollinator landings, whereas PR treatment had no significant effect (Fig. 3a, Table S1). Display size had a significant positive effect on the number of visits in all inflorescence types (Fig. 3a, Table S1). In addition, successive probes within the inflorescence significantly decreased with the R treatment but not with the PR treatment (Fig. 3b, Table S1).

Direct observation revealed that the R treatment had no significant effect on the number of pollinator approaches but significantly decreased landings after the approach (Fig. 4). We found no significant differences in visit duration among and between inflorescence types for both time-lapse camera and direct observations (Table S1). Display size had no significant effect on the landing to approach ratios compiled in our direct observation dataset (Table S1).

REPRODUCTIVE SUCCESS

Fruit set significantly decreased with the R treatment and significantly increased with display size (Fig. 5a, Table S1), whereas the number of seeds per fruit was not influenced by either the treatment or display size (Fig. 5b, Table S1). The number of remaining pollen grains significantly increased with the R treatment and significantly decreased with days after opening (Fig. 5c, Table S1). Display size did not influence the remaining pollen number (Table S1).

Discussion

Our results demonstrate that inconspicuous petals have a landing-site function that enhances reproductive success, rather than a visual attraction function in the scent-attracting plants, thereby supporting the predictions from the hypothesis (Okuyama, Kato & Murakami 2004; Endress & Matthews 2006). We found that pinnately branched petals significantly facilitate the landing of fungus gnats, whereas they are not significantly involved in either visual attraction to the flowers, or the visit duration of pollinators. According to the direct observation results, more than 30% of pollinators failed to land on flowers after approaching completely petal-removed inflorescences, whereas most pollinators successfully landed after approaches to unmanipulated inflorescences. Partial petal removal did not reduce landing frequency, further supporting the notion that the decrease in landings on completely

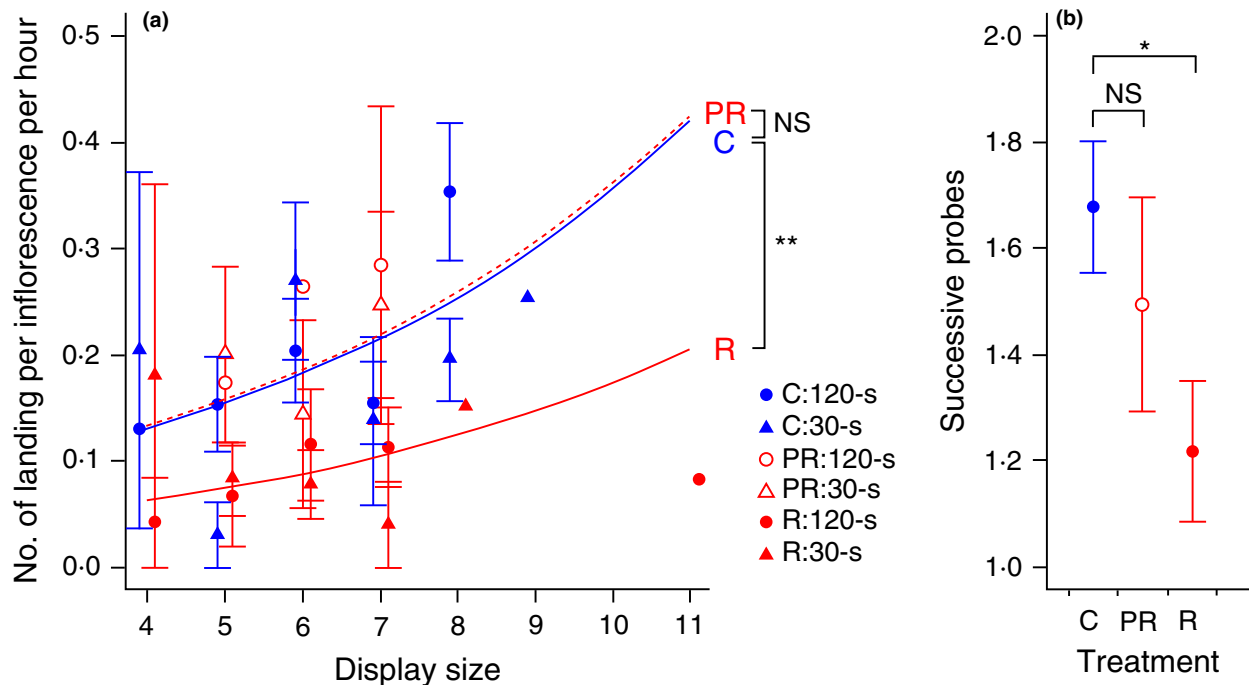


Fig. 3. The results of time-lapse camera photography: relationships between display size (number of flowers per inflorescence) and the mean number (\pm SE) of visits per inflorescence per hour (a); effects of petal removal on the mean successive probes (\pm SE) in the inflorescence (b). Wald test results: NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$. Complete petal removal (R), partial petal removal (PR), unmanipulated control (C).

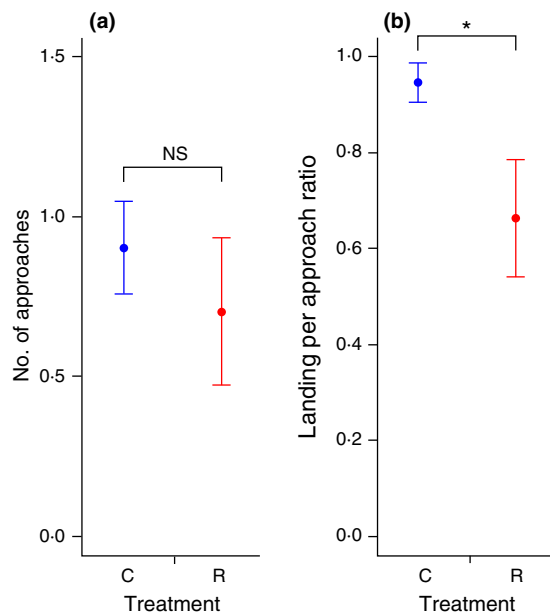


Fig. 4. The results of direct observation: effects of complete petal removal on the mean number (\pm SE) of approaches per 90-min observation period (a); effects of complete petal removal on the landing to approach ratio (b). Wald test results: NS, $P > 0.05$; * $P < 0.05$. Complete petal removal (R), unmanipulated control (C).

petal-removed inflorescences was attributable to diminished landing-site function rather than petal injury.

Complete petal removal significantly decreased both female and male reproductive success, although it did not

influence the number of seeds per fruit. Decreases in fruit set and pollen removal are most likely due to the reduced landings caused by petal removal; however, petal removal did not decrease the number of seeds per matured fruit or the visit duration, which might be autocorrelated (c.f. Manetas & Petropoulou 2000). Thus, the negative impact of complete petal removal on female and male success could be explained by loss of the landing-site function of petals. However, our findings should be interpreted with some caution because the petals of some *M. pauciflora* individuals emit β -caryophyllene (Okuyama 2016). If low concentrations of the volatile emissions attract fungus gnats over short distances, the observed decreases in landings following petal removal may have been due to reductions in β -caryophyllene emission (note that petal removal causes no large changes in floral scent composition). The role of β -caryophyllene as an olfactory attractant for fungus gnats has yet to be fully explored; this would be a promising avenue of future research (Okamoto *et al.* 2015; Okuyama 2016).

Although some petals of fungus-gnat pollinated flowers are thought to have a visual attraction function (Blanco & Barboza 2005; Gaskett 2011), our results do not support this idea. Many studies revealed that floral scent plays an extremely important role in the attraction of fungus gnats (Woodcock *et al.* 2014; Okamoto *et al.* 2015). In *Mitella* species, pale-coloured petals have less attraction area than brightly coloured petals in close relatives, such as *Lithophragma* species pollinated by other insects (Okuyama, Pellmyr & Kato 2008). The closely related species *Tolmiea*

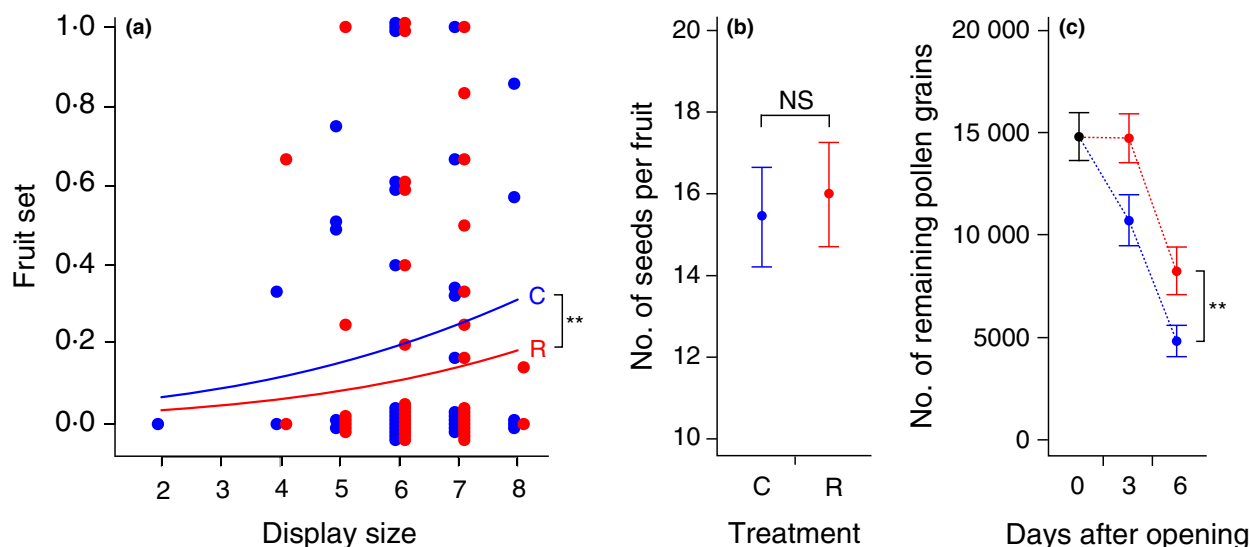


Fig. 5. Effects of petal removal on female and male success: relationship between display size (number of flowers per inflorescence) and fruit set (a). (R), complete petal removal; (C), unmanipulated control. To prevent overlapping of data points (especially when fruit set was zero), small increments (+0.01 or −0.01 for points on the y coordinate when points overlap; −0.08 and +0.08 for points on the x coordinate of C and R respectively) have been added; effect of petal removal on the mean number of seeds per fruit (b); effect of petal removal on the mean number of remaining pollen grains (c). Black circle indicates number of pollen grains remaining in a newly opened flower. In b and c, SE was indicated for each mean value. Wald test results: NS, $P > 0.05$; ** $P < 0.01$.

menziesii is also fungus-gnat pollinated and has dull-coloured filiform petals (Goldblatt *et al.* 2004). The reduced petal area in the genera *Mitella* and *Tolmiea* might have evolved via relaxed selection favouring visual attraction.

In contrast, petals enhanced successive probes within *Mitella* inflorescences, likely resulting in geitonogamous self-pollination (Harder & Barrett 1995), which could reduce both female and male reproductive success. Geitonogamy would result in seed and pollen discounting (Lloyd 1992) and reduced fitness of offspring due to inbreeding depression (Charlesworth & Charlesworth 1987). The presence of petals facilitated successive probes likely by reducing inter-flower distances within *Mitella* inflorescences (c.f. Iwata *et al.* 2012; Fig. 2). However, the average number of successive probes was <2.0 even in control inflorescences, suggesting that the impact of increased geitonogamy on fitness might not be strong in *M. pauciflora*.

Inflorescences with larger displays (i.e. with more flowers) received more pollinator landings and had higher fruit sets, which is consistent with other findings in visually attracting flowers (e.g. Ohashi & Yahara 2001; Iwata *et al.* 2012). Larger displays may release more floral scent or be more visible than smaller displays. In addition, larger inflorescences probably provide more landing places (more flowers and elongated inflorescence stems) for fungus gnats. In contrast, no display size effect on successive probes was found in *M. pauciflora*, unlike in many plants that are bee-pollinated (Ohashi & Yahara 2002). Thus, larger displays appeared to have only positive effects on reproductive success in *M. pauciflora*. No effects of display size on pollinator attraction and landings were detected in

our direct observations (3–7 flowers per inflorescence), likely because we observed a smaller range of display sizes than we did when examining with time-lapse cameras (4–11 flowers per inflorescence).

In conclusion, we have demonstrated that the pinnately branched petals of *M. pauciflora* predominately function as landing sites, and this is the first study to quantify the function of inconspicuous petals in the pollination process in fungus-gnat pollinated flowers. Pinnately branched or filiform petals occur in diverse plant taxa (Endress & Matthews 2006). Several hypotheses have suggested their potential functions (including a landing-site role), but they have not been explored empirically (Endress & Matthews 2006). The provision of pollinator landing sites is one of several petal functions that are not related to visual attraction. The relative importance of these functions is difficult to assess, and they have been little studied, largely because petals often have multiple roles that are not easily disentangled (Endress & Matthews 2006; Whitney *et al.* 2011). Floral and inflorescence traits may have evolved as trade-offs among different functions, including pollinator attraction via visual and olfactory cues, provision of landing sites and regulation of pollinator behaviours on flowers or inflorescences (c.f. Iwata *et al.* 2012). Further studies to discriminate the multiple roles of petals will enhance understanding of the functional properties of these important floral organs; progress will be enabled through investigations that make use of floral mutants, appropriate experimental procedures and/or detailed pollinator observations. These studies will help explain the diversity of petal morphology in flowering plants.

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Data accessibility

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.4gg73> (Katsuhara, Kitamura & Ushimaru 2017).

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Supporting Information

Details of electronic Supporting Information are provided below.

Fig. S1. Fruit set (\pm SE) in *Mitella pauciflora* in three hand-pollination treatments.

Fig. S2. Pollinating fungus gnats on unmanipulated controls.

Table S1. Results of generalised linear mixed model (GLMM) analyses.