Plasticity in flower size as an adaptation to variation in pollinator specificity

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Abstract. 1. Mutualisms, including plant-pollinator interactions, are an important component of ecosystems. 2. Plants can avoid the costs of variation in pollinator benefit by maintaining specificity. 3. We hypothesise a novel mechanism to ensure specificity, which takes advantage of the cognitive abilities of specific pollinators to exclude non-specific flower visitors. 4. Inflorescences of the tropical vine genus Psiguria produce flowers at regular intervals, with subsequent flowers smaller than predecessors. 5. The principle pollinators, Heliconius spp., possess an excellent spatial memory. 6. Therefore, decreasing flower size may ensure specific pollination: once Heliconius individuals have learnt the location of an inflorescence they will return, but inconspicuous flowers should reduce visits by non-specific pollinators with poorer spatial memories. 7. We tested the predictions of this hypothesis with field experiments in Panama. We confirmed that flowers on inflorescences are smaller than their predecessors. 8. Paired experiments showed that larger flowers attracted more pollinators and that the presence of an initial large flower increased subsequent visitation by Heliconius spp. to small flowers, indicating learning behaviour. 9. These results suggest that learning behaviour and decreasing flower size maintain visits from specific pollinators while reducing those from non-specific pollinators. We propose this as a novel mechanism for promoting pollinator specificity and discuss its ecological significance.

Key words. Coevolution, Heliconius, learning, mutualism, plasticity, pollination.

Introduction

Mutualisms, such as plant-pollinator interactions, are a crucial part of highly biodiverse tropical ecosystems, playing an important role in determining ecological dynamics and constructing networks of interdependent species. While these interactions have generally been considered as involving pairs of species, each benefitting the other (Stanton, 2003), multiple interspecific interactions are common, with different costs and benefits to each party (Young, 1988; Stanton, 2003). It may therefore be adaptive for a mutualist to filter out relatively undesirable partners, to ensure specificity with tightly coevolved species (Stanton, 2003; Grangier et al., 2009).

The ability to filter out less beneficial visitors may be hindered if both more and less desirable individuals are attracted to the same stimuli. For example, many angiosperms attract specific pollinators with attractive flowers, but these often simultaneously attract other species (Rosas-Guerrero et al., 2014). Such problems can be mitigated by adaptations to maintain pollinator specificity, such as modified floral morphology, which complements and may coevolve with pollinator morphology (Rosas-Guerrero et al., 2014). Another avenue to maintaining specificity may be temporal plasticity in flower morphology and development, particularly if desirable pollinators have particular behavioural or cognitive capacities, such as learning abilities.
A tightly coevolved interaction can be observed between Psiguria vines and their Heliconius butterfly pollinators (Jiggins, 2016). Psiguria (family: Cucurbitaceae) is a monoecious genus of tropical vine (Murawski & Gilbert, 1986). Psiguria warscewiczii has inflorescences on pedunculated racemes on which orange flowers are produced (Murawski, 1987). Inflorescences typically produce one open flower at a time, which falls off after one or two days, leaving a scar on the peduncle, and is replaced by a new flower (Murawski, 1987). Previous observations have suggested that replacement flowers are smaller than their predecessors (Jiggins, 2016; Gilbert pers. comm.); in effect, flower size decreases over time.

P. warscewiczii is pollinated primarily by butterflies of the genus Heliconius, which have several pollen-feeding adaptations, including traplining behaviour, in which food source locations are visited repeatedly (Murawski & Gilbert, 1986; Murawski, 1987; Jiggins, 2016). Support for learning of food-plant locations includes the experience-dependent mushroom body expansion (Heisenberg et al., 1985; Capaldi et al., 1999; Montgomery et al., 2016) which is evolutionarily coincident with the advent of pollen feeding (Sivinski, 1989), experiments in captivity (Swihart & Swihart, 1970; Dell’Aglio et al., 2016), and mark-recapture experiments (Gilbert, 2014, 1993; Mallet, 1986).

The selective advantage to decreasing flower size may be related to the learning ability of Heliconius spp. Typically, small flowers are less effective at attracting pollinators (Harder & Johnson, 2009). However, Heliconius can potentially remember locations of inflorescences after visiting large flowers, which could reduce the costs of reduced flower size. This could also provide the benefit of reducing visitation by non-specific pollinators, reducing the loss of expensive pollen (Wheelwright & Orians, 1982; Rameau & Gouyon, 1991; Lewis, 1993). Thus, temporal plasticity in flower size could mitigate the costs of attracting pollinators using the same cue.

Here we aim to test whether the inflorescence plasticity can adaptively maintain specific pollination. First, we quantify decrease in flower size. We then test whether both Heliconius and non-specific pollinators are attracted more to larger flowers. We also test for learning behaviour in both Heliconius and non-specific pollinators, with our hypothesis predicting learning in the former and not the latter.

**Materials and methods**

All experiments and observations took place in Gamboa, Panama during June–August 2015 (JR; TT), 2016 (TD; KB) and 2017 (SQ; RS).

**Change in flower size over time**

Psiguria warscewiczii inflorescence age and flower size were measured in the Smithsonian Tropical Research Institute insectaries in July–August 2015. Scars were counted in situ: each scar was interpreted as a two-day addition to inflorescence age. Flower diameter was measured to the nearest 0.1 mm using calipers. We obtained 211 measurements from 123 inflorescences from three different plots.

**Effects of flower size and learning on pollinator visits**

Paired experiments were carried out at rainforest edge, with two potted non-flowering Psiguria vines on greased stands to prevent access by leafcutter ants. The vines were over ten metres apart, and not within sight of each other. Single flowers were placed in Eppendorf tubes half-full of water, which were hung from the vines.

We allocated treatments to vines randomly, and observed from over two metres away. A visit was recorded if a pollinator was seen to feed from the flower. Each feeding attempt was considered unique unless an individual was seen returning to the flower, in which case only one visit was recorded. We avoided mark-recapture methods to identify individuals, as handling effects can reduce the likelihood of them returning to the same location and would have reduced our sample size (Mallet et al., 1987). However, previous findings demonstrating traplining behaviour and site fidelity to home ranges in Heliconius butterflies (Turner, 1971; Murawski & Gilbert, 1986) suggest that individuals do indeed return to the flowers to feed, creating opportunities for learning. Experiments were carried out for 2 h within the period 07:00–09:30, but not during rain, because many pollinators are inactive in rain (TD, personal observation). Non-specific pollinators were identified at least to order level.

To test whether pollinators prefer larger flowers, we placed a large flower (>15.8 mm diameter) on one vine and a small flower (11.3 ± 0.2 mm) on the other. These flowers were the size of a day 1 and day 7 flower, respectively (sizes given in Table S1; based on observation 1). Twelve experiments were performed in 2016.

To test for learning, we placed flowers, of successively smaller sizes on the ‘test’ vine for 7 days (sizes given in Table S1; based on observation 1). Thus, the test vine mimicked the natural state of flowers decreasing in size over time. The ‘control’ vine was placed without flowers until day 7, when a day-7-sized flower was placed there and visits of pollinators to both vines were recorded on day 7. Learning the location of flowers is only possible with test vines. Twenty-eight paired experiments were performed (8 in 2016; 20 in 2017).

**Statistical analyses**

To measure the decline in flower size, we used linear mixed-effects models. As random effects, we included either plot ID (for models using all data) or both plot ID and inflorescence ID (for models considering inflorescences with 20 scars or less), based on model comparison using the Akaike Information Criterion (Akaike, 1974). Inspection of a Q-Q plot showed that residuals were normally distributed except for one outlier (size = 20.4 mm, scars = 2). This point was excluded, and the analysis repeated.

We used generalised linear mixed models with a Poisson distribution to test for differences in visit rate to different treatments and by different species. Species (Heliconius or not-Heliconius),
choice (control/test or large/small), an interaction term between species and choice, and in the case of experiment 1, year (2016 or 2017) were included as fixed effects, with experiment ID a random effect. Nonsignificant terms (identified using likelihood ratio tests) were progressively removed from the model using a backwards stepwise elimination approach.

R version 3.5.1 was used to analyse the obtained data (R Development Core Team), with the packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017).

Results

Change in flower size over time

There was a significant negative correlation between number of scars and flower size on inflorescences of *P. warscewiczii* (slope = −0.02615, n = 211, d.f. = 160, P = 0.009885). Each scar represents a single flower, typically produced daily, so the number of scars is a reasonable indication of inflorescence age. Excluding a single outlier did not affect the relationship (slope = −0.021709, n = 210, d.f. = 162, P = 0.03067, Fig. 1). The effect was stronger for inflorescences up to 20 scars (slope = −0.65282, n = 140, d.f. = 137, P < 0.001, outlier excluded, Fig. 1).

Effects of flower size and learning on pollinator visits

Most butterflies observed at experimental flowers were *Heliconius* spp., primarily *Heliconius erato* and *Heliconius melpomene*, with occasional visits by *Heliconius hecale*. Non-specific pollinators included butterflies from the families Hesperiidae and Nymphalidae, mostly *Anartia Fatima*. There were two visits from Hymenopterans and one from a Dipteran.

We tested whether larger flowers received more visits. Visit rate was higher for *Heliconius* than other pollinators ($\chi^2 = 25.6, n = 12, d.f. = 1, P = 4.21 \times 10^{-7}$) and to large flowers ($\chi^2 = 39.9, d.f. = 1, P = 2.68 \times 10^{-10}$) (Fig. 2a). The interaction term was non-significant, suggesting no difference between *Heliconius* and other pollinators in preference ($\chi^2 = 0.484, d.f. = 1, P = 0.487$).

We investigated whether the presence of a large flower increased subsequent visitation to a small flower. Visit rate was higher for *Heliconius* spp. than other pollinators ($\chi^2 = 154, n = 28, d.f. = 1, P = 2.20 \times 10^{-16}$). When flowers of decreasing size were placed at a location, this increased visits by *Heliconius* as compared to the control ($\chi^2 = 54.0, n = 28, d.f. = 1, P = 2.08 \times 10^{-13}$), implying that *Heliconius* butterflies learn the location of food sources (Fig. 2b). The year during which experiments were done did not significantly influence visit rate ($\chi^2 = 2.38, n = 28, d.f. = 1, P = 0.123$). Despite the number of visits by non-specific pollinators to test and control treatments being equal and an interaction plot suggesting a difference in learning ability, the low visitation rate led to a non-significant interaction term, suggesting no difference between *Heliconius* and other pollinators in preference ($\chi^2 = 1.90, n = 28, d.f. = 1, P = 0.168$). A statistical power analysis using effect sizes calculated from a model including the interaction term found that for $n = 28$, the interaction term was non-significant in 52% of 10000 iterations. It may therefore require more experiments to form conclusions about the extent of learning by non-specific pollinators.
Fig. 2. (a) The number of visits by Heliconius and non-specific pollinators to small and large flowers. Large flowers were visited more frequently, and visit rate was higher for Heliconius. (b) The number of visits by Heliconius and non-specific pollinators to flowers of the control treatment (learning not possible) and flowers of the test treatment (learning possible). Visit rate was higher for Heliconius and to the test treatment. See text for test statistics.

Discussion

Species interactions in tropical ecosystems lead to ecological stability and maintenance of diversity. We have studied a classic example of coevolution and demonstrated the potential for pollinator learning to influence inflorescence morphology and plasticity, and a mechanism by which a plant can filter out less beneficial mutualists.

In P. warscewiczii, flower diameter decreases with inflorescence age, with most of the change occurring during the first 40 days. We have shown that pollinators are more likely to visit larger flowers. We have also demonstrated that small flowers receive more visits when Heliconius butterflies are given the opportunity to learn their location through prior placing of larger flowers. These results support our proposed mechanism by which pollinator specificity is promoted by temporal plasticity in flower size and the learning ability of Heliconius spp., although they do not rule out other putative benefits of decreasing flower size, such as a reduction in energy expenditure when producing later flowers.

One limitation is the scarcity of data on non-specific pollinators, which prevents us from demonstrating reduced learning capabilities in non-specific pollinators compared to Heliconius. This low sample size for non-specific pollinators in the learning experiment matches the expectations based on our hypothesis: we expected few non-specific pollinators on the test vine (since unlike Heliconius butterflies, they cannot learn the vine location), as well as few pollinators of any specificity on the control vine (since small flowers prevent pollen wastage by attracting few pollinators). At present, we cannot distinguish this explanation for the scarcity of data on non-specific pollinators from mere differences in pollinator abundances. Future studies could investigate the changes in the proportion of specific versus non-specific pollinators with decreasing flower size.

We echo Stanton’s (2003) argument that studying variation in the specificity and benefit of pollinators is essential to understanding adaptations and consequences of pollination syndromes. We suggest that for P. warscewiczii the costs of attracting multiple pollinators with a single stimulus have been mitigated by plasticity in the plant and the learning ability of pollinators. This provides an example of adaptation to variation among interspecific interactions.

There is considerable evidence of natural selection on flower attributes which increase the likelihood of attracting suitable pollinators (Rosas-Guerrero et al., 2014), yet few known examples of specific adaptations of angiosperm inflorescences (Harder et al., 2004). Our study suggests that selection may also act on attributes, which limit the attraction of undesirable
pollinators. Such pollination specificity can have wide-ranging ecological consequences by allowing the persistence of species, which are typically at low population densities and therefore require specific pollination (Baker & Hurd, 1968; Janzen, 1971), such as *P. warscewiczii* (Murawski & Gilbert, 1986).

Investigating plant-pollinator interactions can have broader implications for our understanding of biodiversity and the ecological complexity: such interspecific interactions create variation in biotic niches, promoting biodiversity (Bawa, 1990). Learning can result in behavioural plasticity that may facilitate speciation, perhaps through species segregation in micro-habitat use (Estrada & Jiggins, 2002; Merrill et al., 2015). While such interactions can increase stability as well as biodiversity, they can also result in extinction cascades from the local loss of participating species, with potentially far-reaching effects (Bond, 1994). This is especially true when there is a high degree of dependency between species such as in specific plant-pollinator interactions (Bawa, 1990; Burkle et al., 2013). Furthermore, as pollinator specificity allows persistence of plants at low population densities, extinction risk from habitat fragmentation is increased (Lienert, 2004). Therefore, the evolution of adaptations to biotic variation may have a range of effects on biodiversity and ecosystem stability.

Our study suggests a potentially unique mechanism by which a plant can minimise pollen wastage: by attracting pollinators with a good spatial memory and avoiding non-specific pollinator visits through decreasing flower size. While the evolutionary benefit of this is clear, the broader ecological consequences of such interactions may be complex and unpredictable in the face of a changing world.

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**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Supporting Information**

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

**Table S1.** The size of flowers used for each day in the learning experiment.

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