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Do Beetles Prefer the Odor of Female-Stage to Male-Stage Flowers in Atemoya, a Cantharophylous Protogynous Fruit Tree (Annonaceae)?

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ABSTRACT

The flowers of annonaceous fruit trees cherimoya (*Annona cherimola*) and atemoya (*A. cherimola* x *squamosa*) exhibit protogynous dichogamy. Their main pollinators are sap beetles (Coleoptera: Nitidulidae). However, pollination by beetles is usually not sufficient for commercial fruit production, so costly hand pollination is required in many areas. These beetles on cherimoya and atemoya are thought to visit the female-stage flower, remain inside it, and leave the flower when it has transitioned to the male stage; however, no study has yet directly elucidated the visiting behavior of the beetles. In this study, we examined this hypothesis using olfactometer testing in the field. Both male and female sap beetles, *Carpophilus marginellus*, were significantly attracted to the odor of female-stage flowers, but not to the odor of late male-stage flowers. We conclude that the beetles prefer the odor of female-stage flowers to that of late male-stage flowers. These findings support the above hypothesis describing beetle pollination behavior.

Key words: Annonaceae, Nitidulidae, floral odor, beetle pollination, behavior.

INTRODUCTION

The custard apple, or cherimoya, *Annona cherimola* Miller, the sugar apple, *A. squamosa* Linnaeus, and their hybrid, atemoya (*Annona cherimola* x *squamosa*) are cultivated for their fruits, especially in warm regions, worldwide. Cherimoya originates from the highlands of Peru, whereas the sugar apple originates from Caribbean islands. They belong to the family Annonaceae, a mainly tropical plant taxa of the order Magnoliales, which is relatively primitive in angiosperm (Moore *et al*., 2007). A majority of studied plants of the family exhibit protogynous dichogamy and employ beetles as pollination agents (Gottsberger, 1974; 1988; 1989a; 1989b; Armstrong and Irvine, 1989; Andrade *et al*., 1996; Dieringer *et al*., 1999; Bernhardt, 2000; Endress, 2010). *Annona* fruit trees are also protogynous and known to be cantharophilous(González and Cuevas, 2011). The timing of the change of sex from female to male varies with locality and variety (Nadel and Peña, 1994; González and Cuevas, 2011; Kishore *et al.,* 2012). In Israel, atemoya flowers enter the female stage in the evening, and transition to the male stage approximately 24h later (Podoler *et al*., 1984). On the other hand, in Florida, atemoya flowers open in mid- to late afternoon, and become male around noon the next day (Nadel and Peña; 1994). González and Cuevas (2011) report that in Spain, cherimoya anthesis occurs gradually from early afternoon, the flowers transition to the male stage 24h later. In Japan, the sexual phase usually changes from 16:00 to 21:00 in both cherimoya and atemoya, although a small proportion of atemoya flowers become male in the morning. Because the female and male stages do not normally overlap, natural fruit set without pollinators is generally low (Richardson and Anderson, 1996).

As is usual in the family Annonaceae (Okada, 1993), *Annona* flowers have no nectary. In addition, they have green petals, and only small openings between the petals during the female stage (Podoler *et al.*, 1985; González and Cuevas, 2011). As a result, attempts to use honeybees or bumblebees for pollination have failed, leading to the need for costly hand pollination in places where natural fruit set is not sufficient (Saavedra, 1977; George and Campbell, 1991; Richardson and Anderson, 1996). The long search for effective pollination agents continues in agricultural scene.

Surveys on natural pollinators for *Annona* fruit trees have shown that the flowers rely mainly on beetles of the family Nitidulidae (Gazit *et al.*, 1982; George *et al.*, 1989; Nadel and Peña, 1994; Nagel *et al.*, 1989; Blanche and Cunningham, 2005; Jenkins *et al.*, 2013; but see also Caleca *et al.*, 1996; 1998) for pollination. A periodical collection of flower visitors in sugarapple and atemoya orchard in Florida suggested that insects are attracted to female-stage flowers, and remain there until the flowers become male (Nadel and Peña, 1994). The insects are assumed to be released from the flower with pollen grains adhering to their body surface (Fig. 1; Gazit *et al.*, 1982). At this time, new flowers enter the female stage to which the insects with pollen are attracted, and pollen grains will in this way be deposited on their stigmata. This hypothesis is accepted in many studies. However, the pioneer work of Podoler *et al.* (1984) showed that experimentally cut atemoya flowers attracted beetles in the morning and evening at the female and the early male stage, respectively, and that they lose their attractiveness in the late male stage. This does not necessarily support the hypothesis, because when the late male-stage flowers have lost their odor, female-stage flowers may also have lost their odor. Thus, the movement of beetles from male- to female-stage flowers may be prevented. However, until now, no study has observed the actual behavior of the beetles; i.e., whether they prefer female-stage flowers to late male-stage ones in these fruit trees.

Although there is no evidence that flowers offer mating site for beetles in chrimoya, atemoya and sugarapple (Peña *et al.*, 2002), many beetle-pollinated flowers offer mating site for the visitors (e.g., Thien *et al.*, 1985; Tang, 1987; Johnson and Midgley, 2001). In addition, males of many nitidulid beetles especialy in the genus *Carpophilus* produce aggregation pheromones which attract both sexes. Therefore, even if only the male beetles was attracted to the flower odor, both sexes can be in the flower because the male individuals attract another individuals of both sexes by the aggregation pheromone. However, this scenario has not been tested in previous field studies.

Tsukada *et al.,* (2005) reported 29 species of insects from cherimoya flowers in orchards in Wakayama Prefecture, Japan. Of these, 7 species were nitidulid beetles. Higuchi *et al.* (2014b) studied the pollination ability of orchard-caught beetles, and concluded that *Haptoncus ocularis* (Fairmaire) (Nitidulidae) and *Mimemodes monstrosus* (Reitter) (Rhizophagidae) can pollinate cherimoya. Further, Higuchi *et al.* (2014a) revealed mass-reared *H. ocuralis*, *M. monstrosus*, and *Carpophilus marginellus* Motschulsky (Nitidulidae) to have the ability to pollinate cherimoya, even if reared exclusively on pineapple for several generations. These insects therefore retain the ability to respond to the odor of cherimoya flowers. In the present study, we investigated whether the female- and late male-stage flower at the same time of a day attract *C. marginellus* adults so that the pollen can be transferred from male-stage flowers to female-stage ones on atemoya trees. Furthermore, we sexed the tested beetles to test if only male or both sexes respond to the odor.

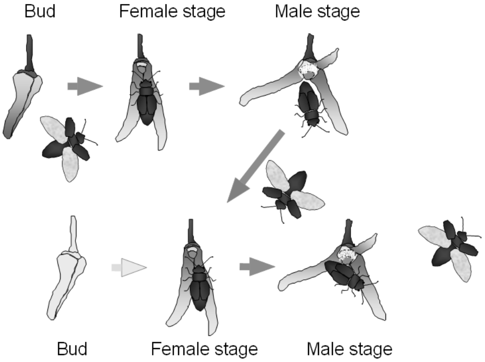

Fig. 1. Flowering and the hypothesized pollination pattern by beetles in *Annona* spp.

MATERIALS AND METHODS

Study sites and materials

Atemoya trees were cultivated at the Kinan Fruit Tree Science Branch of Mie Prefecture Agricultural Research Institute, Mie, Japan, and in a commercial orchard on Ishigaki Island, Okinawa, Japan. These trees were used for *in situ* preference experiments in the summer of 2007, from June to August in Mie, and in November in Okinawa. The Mie site comprised five atemoya trees planted in a greenhouse. The Okinawa site was an open orchard, with 13 atemoya trees planted alongside other fruit trees. The atemoya flowers in our study sites emitted a fruit-like odor in the evenings, during both the female and male stages. In some cases, the flowers emitted odor even in the morning.

Laboratory stock of the sap beetle *C. marginellus* was used for the preference test. This stock was founded from individuals caught in atemoya flowers on the campus of Mie University, Tsu, Mie, Japan. They were kept at 25 ˚C, 16L:8D conditions with cut pineapple as food. See Tsukada *et al.* (2008) for details of rearing and larval development.

The insects were prepared as follows. 1) Last-instar larvae were selected from the stock. 2) These larvae were individually kept in small vials until eclosion, with wet tissue paper providing the pupation site. 3) Newly-emerged adults were supplied with water for 3 - 5 days, the required period for sexual maturation, until the experiment.

Experiments

T-shaped glass tubes, 8 mm inner diameter, 70 mm in length (the vertical part of the T) and 110 mm wide (the horizontal part of the T) were connected with transparent vinyl chloride tube (12 mm inner diameter) at the three ends (Fig. 2). The vinyl tube from the lower end of the glass tube was connected to an air pump (Shibata Mini Pump MP-2N), which vented the air from the tube. This vinyl tube was readily disconnected from the T-tube to allow placement of a test insect in the tube.

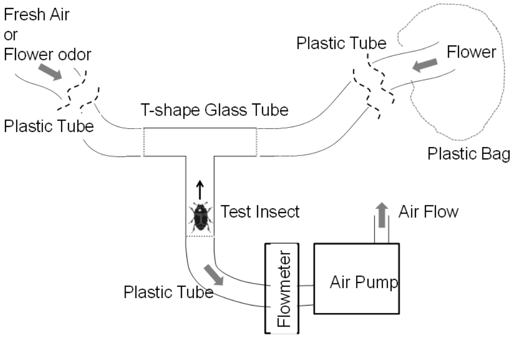

Fig. 2. Outline of the T-shape olfactometer. Insects were individually placed in the T-shape glass tube and allowed to choose one of the two ends.

Contrary to the results of Podoler *et al.* (1984), who carried out preference experiments using cut atemoya flowers, our preliminary experiments showed that cut flowers have a repellant effect on the beetles. We therefore did not cut them: instead, a flower on a tree was gently covered with a plastic bag measuring ca. 30 x 20 cm. Openings on the bag allowed ventilation. One of the vinyl tubes from an upper end of the glass tube was connected to the flower on the tree. The last tube was led to outside of the greenhouse as a control or was connected to another flower.

The experiments were always carried out from 19:00 - 22:00, after sunset. The ambient temperature was 20 - 26 ˚C. We expected the pollinator beetles to move from late male-stage flowers to a female-stage flowers in this period because during this time, the male-stage flower gradually loses its odor, whereas the female-stage flower may still emit a fruit-like odor. Previous studies have suggested that both female and male-stage flowers emit a fruit-like odor (Podoler *et al.*, 1984), and beetles which entered during the female stage leave the flower late after the flower enters the male stage (Nadel and Peña, 1994). Our preliminary observations supported these observations.

Do Beetles Prefer the Odor of Female-Stage to Male-Stage

Combinations of female-stage flower vs. control, male-stage flower vs. control, and female-stage flower vs. male-stage flower were tested for beetle attraction. The flow of air was regulated, using flow meters, at 0.5 - 0.7 l / min at the lower end of the T-tube. Insects were gently placed in the T-tube from the bottom individually, and observed for 5 min. When the insect reached one of the upper ends of the T-tube, their choice was recorded and the trial ended. All the flowers and insects were used for the experiment only once. The number of trials with each combination was about 20. The tubes were replaced every time and carefully washed with water and ethanol, then dried naturally before the next use. Voucher specimens are deposited in the Insect Ecology Laboratory of Mie University. The obtained data were analyzed using a binomial test for the difference within the sex of beetle, and by Fisher’s Exact test for the difference in behavior between the two sexes of the beetle. The tests were performed with R 2.9.2 software (R Development Core Team, 2010).

RESULTS

In five of the total of 117 trials, the insects did not choose either of the two ends of the T-tube within 5 min. In consideration of this relatively small number, we omitted them from the analyses.

Both male and female individuals were significantly more likely to choose female-stage flower to controls (Fig. 3; $p < 0.01$, $p < 0.001$, respectively). Although male insects made more definitive choices than did the females, there was no significant difference between male and female insects.

For male-stage flowers, both male and female insects showed no statistically significant choices (Fig. 4). Although male insects were slightly more likely to choose flowers, while female insects were more likely to choose the control, there was no statistically significant difference in this behavior between the two insect sexes.

Finally, for the test between the flowers in the two stages, female-stage flowers were chosen over male-stage flowers by both male and female insects (Fig. 5; $p < 0.001$, $p < 0.05$, respectively). There was no difference in this behavior between the two sexes of the insect.

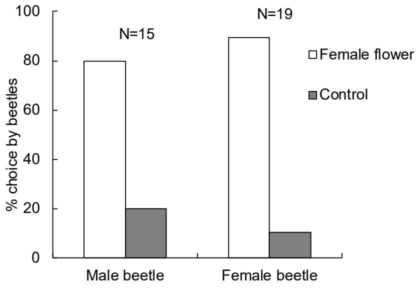

Fig. 3. Results of the choice test by male and female *C. marginellus* to the odor of a female flower or control.

Fig. 4. Results of the choice test by male and female *C. marginellus* to the odor of a male flower or control.

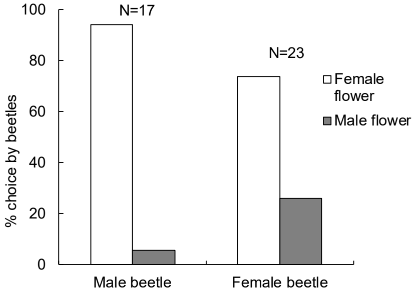

Fig. 5. Results of the choice test by male and female *C. marginellus* to the odor of a female flower and a male flower.

DISCUSSION

Our results clearly show that *C. marginellus* adults are attracted to the odor of female-stage flowers of atemoya in the evening, but at that time, they are not attracted to the odor of late-male-stage flowers. These results are consistent with the previous hypothesis, that in the atemoya and its relative's pollination system, beetles are attracted to the flower during the female stage, remain in the flower, and are released during the male stage with pollen grains adhering to the surface of their body. The released beetles are then assumed to visit a new female-stage flower (Nadel and Peña, 1994). However, early male-stage flowers emit a strong odor that can also attract beetles (Podoler *et al.*, 1984). Furthermore, Nadel and Peña (1994) noted that beetles do not escape from the male flowers immediately after the sexual phase change. Our preliminary observations showed the same results (unpublished). Nevertheless, this study has shown the conventional hypothesis of pollination for *Annona* fruit trees to be most likely true. Even though a floral odor is emitted from female and male-stage flowers from evening to early night, the timing of the disappearance of the odor might be slightly different; i.e., emission of odor during the female stage lasts longer, whereas that during the male stage ceases sooner. This difference appears to motivate beetles to move from male-stage flowers to female-stage flowers. Therefore, breeding and/or cultivation techniques that ensure the disappearance of male odor while the female flower emits a strong odor may improve fruit set by beetle pollination.

Some cycad and relatively primitive angiosperms employ a push-pull or similar strategy to control insect behavior and achieve effective pollination (Terry *et al.*, 2007; Teichert *et al.*, 2011). Protogynous flowers typically emit an attractive odor during the female stage and emit a repelling odor at the late female stage, and then emit an attractive odor again in the male stage (Terry *et al.*, 2007). In this study, however, we found no repellant effect of late male-stage floral odor. Instead, the attractiveness of the late male-floral odor was the same as that of the control (ambient air) (Fig. 2). We therefore suggest that the strong odor in the early male stage simply fades with the elapse of time.

The results of this study support the conventional hypothesis on beetle pollination behavior for cherimoya and atemoya (Gazit *et al.*, 1982; Nadel and Peña, 1994). However, atemoya flowers seem to emit their odor usually only in the evening to the early night (Podoler *et al.*, 1984), and occasionally in the morning (Tsukada, unpublished data). The flower apparently provides no rewards such as nectar or warmth. On the other hand, Podoler *et al.* (1985) recorded the longevity of a pollinator, *Carpophilus hemipterus*, to be greater with experience of contact with flowers than without. The reason for this has not been clarified. However, if this is applicable for all visitors, it might be the reason why the beetles stay for long periods in the flower after they have been attracted to the female-stage flowers.

Beetle attraction by floral odor is found also in other genera of the family Annonaceae (Andrade et al., 1996; Bernhardt, 2000; Gottsberger, 1977; 1989a; 1989b; 1999; 2012; Jürgens *et al*., 2000; Maia *et al*., 2012). In *Asimina*, the flower emits an odor that consists chiefly of esters, typical of fruit odors (Goodrich and Raguso, 2009). Since most flower-visiting nitidulid beetles found in *Annona* flowers are frugivorous (George *et al.*, 1992; Tsukada *et al.*, 2005), it is likely that flowers chemically mimic the odor of rotting fruit to lure frugivorous beetles (Peña *et al.*, 2002; Goodrich *et al.*, 2006).

We found no significant difference between the two sexes of beetles in terms of attraction to the floral odor. Therefore, it is unlikely that males visit the flowers first by responding to the odor, and attract other individuals by their aggregation pheromone. Rather, beetles are attracted to the floral odor regardless of their sex. This can be confirmed by examining the distribution of beetles of each sex in flowers, in the field.

In the family Annonaceae, which are relatively primitive angiosperms, beetle pollination is most common, although thrips, flies, cockroaches and other insect species also pollinate certain plant species (Jürgens *et al.*, 2000; Nagamitsu and Inoue, 1997; Saunders, 2012). Molecular phylogeny shows that pollination by small beetles such as nitidulid beetles is an ancestral trait in Annonaceae (Saunders, 2012): in the gymnosperm family Cycadaceae, which is a neighbor species to angiosperms (Schneider *et al.*, 2002), beetles appear to be the most frequent pollinators (Kono and Tobe, 2007; Procheş and Johnson, 2009). Therefore, the sophisticated pollination system seen in *Annona* spp., although not specialized, seems to be common in the early era of angiosperm evolution. However, because atemoya is a hybrid of cherimoya and sugar apple (Morton, 1987) that was developed only a century ago, natural selection is unlikely to have shaped the flowering behavior of this plant. Careful observation of sugar apple and cherimoya should therefore provide more information and lead to a better understanding of the evolution of pollination in *Annona* spp.

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