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ORIGINAL RESEARCH

Mechanical vs. Beetle-mediated Self-pollination in *Gossypium Tomentosum* (Malvaceae), an Endangered Shrub

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Abstract: Experimental hand pollinations of the endangered, Hawaiian, endemic, *Gossypium tomentosum* Nutt. Ex. (Malvaceae) showed that it was self-compatible, but self-pollination resulted in reduced reproductive output. Field observations and pollen tube analyses using fluorescence microscopy showed that mechanical self-pollination in this species included a mechanism known as bending stigmas. A receptive stigma bent backwards and contacted dehiscent anthers in 7% of flowers found on 17 *G. tomentosum* plants. The yellow flowers were nectarless and were not visited by most anthophilous insects *in situ* except for the introduced, nitidulid beetle, *Aethina concolor* Macleay. Collections and insect GI-tract dissections showed that *A. concolor* carried and ate the pollen of the host flower. Field observations recorded regular contact between beetles and stigma lobes as these insects exited the flowers effecting self-pollination. Behavioral experiments showed that the beetles responded positively to a yellow visual cue. Under some circumstances, an introduced pollen vector may help maintain a low level of reproductive success in an insular endemic.

Keywords: Kauai, nitidulid, endemic plant, pollen tube, self-compatible, self-pollination, stigma, Malvaceae

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Introduction

As an estimated 80% of all flowering plants depend on animals for sexual reproduction[1,2] both the decline in pollinator diversity and the implications of that loss have become a serious concern in plant conservation and habitat maintenance.[3–4] Habitat loss and the introduction of invasive species pose a serious threat to plant-pollinator interactions.[3] The pollination systems of declining and/or rare endemic species need greater investigation as their distributions are invaded and overwhelmed by both alien flora and anthophilous and/or anthophagous fauna.

However, when a native pollination system breaks down due to these factors some native plant species may continue to set seed due to two, overlapping reasons. First, many plants found on highly isolated, oceanic islands (e.g. the Hawaiian archipelago) are self-compatible. This can offer a reproductive "fail-safe mechanism" when coupled with mechanical self-pollination in the decline or absence of native animal pollinators. Self-compatibility is especially high in island species because many are derived from self-compatible, and often self-pollinating ancestors,[5,6] as many insular plants are often highly self-compatible they should also exhibit various mechanisms for delayed self-pollination in the absence of pollinators even though out-crossing remains the preferential mode of reproduction to increase genetic diversity and improve reproductive fitness.[7]

Second, although exotic species are usually detrimental to the survival of native species,[8] exotic generalist pollinators can, in some cases, successfully disperse the pollen of native plants. These naturalized animals assume the role of primary pollinator when endemic pollinators decline or become extinct. For example, the Hawaiin *Freycinetia arborea*, remains bird-pollinated although the native honeycreeper birds (Drepanidae) are extinct through the plant's current range. It is now pollinated primarily by exotic but the naturalized Japanese white-eye bird (*Zosterops japonica*).[9]

Gossypium tomentosum (Malvaceae) is yet another rare, Hawaiian endemic. In July 2003, G. Booth and P. Bernhardt observed a naturalized population of this species at the National Tropical Botanical Gardens (NTBG) on Kauai. They observed many, small beetles in the flowers of *G. tomentosum* (Bernhardt and Booth, unpublished). Specimens were all identified by the Bishop Museum as an exotic species, *Aethina concolor* Macleay (Nitidulidae). This beetle is native to Australia and widespread throughout the South Pacific region.[10]

Nitidulid beetles are found worldwide and are attracted to plants that secrete floral nectar and/or produce soft, fleshy fruits.[11] These insects prefer high temperatures at 25–30 °C, and relatively high humidity (75%–95%).[12] Historically, Hawaiian sugar cane and topical fruit companies have regarded this species as a nuisance, but not as a noxious pest.[13,14]

Although small beetles such as nitidulids are recognized as plant pollinators,[15–23] they are usually not recognized as primary pollinators.[24–31] They are considered too small to carry an effective pollen load and have a short flying range. This is believed to limit their value as agents of cross-pollination.[32] Nevertheless, a review of the literature indicates that nitiulids are co-pollinators of some members of the Annonaceae, Arecaceae and Dipterocarpaceae along with beetles belonging to other families (e.g. Cleridae, Curuclionidae, Staphylinidae etc.). Nitidulids are the only known pollinators of some *Amorphophallus*, *Calycanthus* and *Degenaria* species (see review in).[33] However, with the exception of *Drimys* (Winteraceae) nitidulids are not commonly regarded as pollen vectors of angiosperms with generalist pollination systems (see review in).[33]

The objective of this study is to document the floral biology and breeding systems of *G. tomentosum*, a species close to extinction in the Hawaiian Islands. The following questions are addressed: 1) Is *A. concolor* the primary pollinator of *G. tomentosum* at one of its current sites? 2) Is there a mechanism for "fail-safe" self-pollination (mechanical autogamy) in *G. tomentosum*? 3) Does *A. concolor* carry the pollen of *G. tomentosum* and contact receptive stigmas when it visits the flower? 4) Does *A. concolor* receive any rewards from the host flower? 5) How does *A. concolor* find the flowers of *G. tomentosum*?

Methods and Materials

Plant and insect study species

Gossypium tomentosum Nutt. Ex. Seem (Malvaceae), also known by the Hawaiian name "ma'o"[34,35] is the only endemic Hawaiian cotton.[36,37] It was used by ancient Hawaiians as a source of yellow-green dye but not as a source of fiber.[38]

Gossypium tomentosum is a tetraploid species (2n = 52;)[36,37] occuring in scattered, small populations throughout the Hawaiian archipelago. It was recorded as extinct on Kauai[36] until it was reintroduced as two small populations at the National Tropical Botanical Gardens (NTBG), in 1982 by Steve Perlman. All living material for these two populations were imported originally from remnant populations found on the Hawaiian islands of Lanai, Molokai and Kahoolawe.

The species usually grows as a coastal plain shrub 0.5–1.5 m in height.[35,37] The leaves and bracts lack extra-floral nectaries and the flowers lack the floral (nuptial) nectaries on the inner sepal surfaces associated with other members of the Malvaceae. It bears perfect solitary, uniformly yellow flowers approximately 3–4 cm long. As in most members of the Malvaceae, the stamens are adnate to the corolla and form a continuous sheath around the style. The style within the genus *Gossypium* is undivided. Consequently, the stigma of *G. tomentosum* is entire and lacks the five subsessile lobes associated with other members in this genus. The original pollinator(s) of *G. tomentosum* are unknown, but Fryxell[39] and De Joode and Wendel[36] interpreted floral presentation as indicative of pollination by crepuscular insects.

Aethina concolor was originally called *Macroura concolor* Macleay[40] and was first reported in commercial shipping records as a pest in pineapple shipments.[13,14,41] Vouchers of both *G. tomentosum* and *A. concolor* were collected from both study sites at NTBG and deposited with the Brigham Young University Bean Museum.

Study sites

The two populations of *G. tomentosum* at the NTBG on Kauai, Hawaii were selected for both experimental pollination studies and for flower-insect observations. The first population of eight plants of *G. tomentosum* (Site I) was located in the Native Garden at an elevation of 29.6 m. The second population of nine plants (Site II) was located at the Visitors Center approximately 0.8 km away from the first site, elevation 38.1 m. They remain the only known populations of *G. tomentosum* on Kauai, HI. At both sites, flowering occurs annually from late May through late July. Our observations and experiments were conducted from 01/vi/04–09/vii/04.

Floral life-span and rate of fruit set

To record variation in the life-span of reproductive organs we hung labeled jewelers tags on the pedicels of 36 mature flower buds belonging to the 17 plants in both populations. Buds were tagged on five different dates over the flowering period of the species (9/vi/04 n = 8; 10/vi/04 n = 8; 14/vi/04 n = 6; 18/vi/04 n = 8; 24/vi/04 n = 6). From 9/vi/04 through 5/vii/04 we also recorded whether these tagged flowers set seed. Stigma receptivity and stamen dehiscence were determined visually. A receptive stigma was wet and sticky in appearance when examined with a ten power hand lens.

Additional counts of total fruit present on 17 plants at both sites were made over 19 days during the 2004 research season. We collected fruit from both populations twice (n = 34 fruits), dissected them and recorded the number of seeds in each locule. Seeds were classified as either normal or abortive. Physical size and shape were used to discern whether seeds were abortive (i.e. very small and shriveled).

Floral dimensions

Using digital calipers (Mitutoyo SR44), we measured the following dimensions to compare the size of the floral organs vs. the size of floral foragers and prospective pollinators.

1. **Width and height.** We measured the corolla width at its widest point across a fully open flower and the height from the base of the corolla to the tip of the protruding stigma (the highest part of the flower that extends up beyond the edge of the corolla; n = 68).
2. **Corolla span.** To establish the opening times of the flowers, the span of the opening corolla on a flower from each plant was measured in 15-minute increments from 06:45 h, when the *G. tomentosum* buds were all tightly closed until 11:30 h, when the *G. tomentosum* flowers were fully open. These measurements were made on three separate days (n = 23).
3. **Stigma collapse onto dehiscent anthers.** The stigmas of some flowers were observed to collapse onto their dehiscent stamens. The number of "collapsed" stigmas vs. the number of stigmas remaining upright was counted for each plant at varying times of the day. This was repeated for fourteen days throughout the flowering season.

Pollen-pistil interactions

To determine the breeding system of *G. tomentosum* a series of hand-manipulated experiments were carried out using all 17 plants at sites I and II between 18/vi/04–06/vii/04. Flowers were placed in two experimental categories. Hand-manipulated cross-pollinations were always performed using pollen collected from one study site and applying it to stigmas at the other site. We tagged and bagged mature buds the evening prior to the pollination experiments following techniques and protocols of Lipow et al.[42] When a flower opened the following morning it received one of two treatments.

1. **Hand-manipulated self-pollination.** The flower bud was labeled and isolated in a bag of bridal veil netting. When the corolla opened, the bag was removed and the stigma was covered with pollen derived from the same flower. Pollen was applied until it was visible to the naked eye. The bag was then reaffixed for the duration of the experiment.
2. **Cross-pollination.** The flower bud was labeled and isolated as in 1. When the corolla opened the bag was removed temporarily, all stamens were removed and the stigma was hand-pollinated with pollen from a single flower derived from the other population. Pollen was applied to the stigmatic surface until it was visible to the naked eye and then the bag was reaffixed for the duration of the experiment.

Twenty-four hours after each treatment the flowers were picked, the bags removed and the whole gynoecium was fixed in a 3:1 solution of 95% EtOH: glacial acetic acid for two hours. The fixative was then decanted, and the flowers were stored in 70% EtOH.

To count the number of pollen tubes in each pistil, the staminal sheath was removed and the pistil was placed in a small beaker. Specimens were covered with a 10% (w/v) solution of sodium sulfite and autoclaved for three minutes at 121 C. The specimens were then cooled with de-ionized water for 15 minutes. Each pistil was mounted separately on a glass slides, covered with 3–5 drops of decolorized aniline blue, covered with a cover slip, and the softened tissue was spread by tapping the coverslip with the tip of a probe to flatten the pistil and spread the tissues. The slides were labeled and refrigerated a minimum of 24 hours.

A Zeiss Axioskop 2 Plus microscope with a 100 watt fluorescent source was used to view the pollen tubes. The number of pollen grains on the stigma, the number of pollen tubes in the style, and the number of pollen tubes reaching the ovary, were all counted and recorded to determine successful rates of pollination (see Lipow et al).[42]

Analyses of attractants and rewards

Fresh flowers were collected and placed in clean, sealed, glass, scent jars for two hours. The presence of a discernible floral fragrance was recorded by opening the lids and smelling the contents.[43]

To verify whether flowers of *G. tomentosum* always fail to secrete nectar, we sampled floral fluids hourly from randomly selected flowers with expanded corollas (n = 96) from all 17 plants at both study sites. Any liquid present in the flower was collected using a 10 µl capillary tube and placed in a Brix handheld refractometer. The volume of fluid and percentage of sugars present was recorded.

Floral foragers

To identify the primary pollinators, insect visitors to flowers were recorded and collected. A net was first used to capture the insect and then the specimen was placed in a killing jar charged with ethyl acetate. Insects were taken to the lab for pollen load analysis to quantify the amount of pollen being carried by the insect. We recorded observations of physical contact between an insect and the receptive stigmas of *G. tomentosum.* We measured specimens of *A. concolor* with the same equipment used to measure flowers (see above).

To determine whether *A. concolor* pollinated stigmas of *G. tomentosum* we monitored plants at both sites over six weeks. Five randomly selected flowers were examined, on each plant, for the presence of nitidulids. Nitidulid behavior was categorized as copulating, feeding, or resting on the petals. The total number of beetles (n = 2866) and the type of behavior was recorded hourly.

Live nitidulids collected from *G. tomentosum* flowers were placed in a petri dish with *G. tomentosum* pollen and observed over 24 hours to see if they consumed pollen. The observation series was

repeated four times with five nitidulids per Petri dish. The nitidulids were then returned to the plant from which they were collected.

To assess the identity and number of pollen grains carried by each visitor to *G. tomentosum* we made a library of pollen grains from flowering plants within the study site. Dehiscent stamens were placed on glass slides. The pollen was teased out with probes, stained with 1–2 drops of Calbera's fluid to make a semi-permanent mount[33,44] and labeled to species for future reference.

To count and identify the pollen grains carried by the beetles each euthanized beetle collected on *G. tomentosum* was placed on a separate glass slide and washed in a few drops of 70% EtOH. The insect specimen was removed from the slide and the slide was allowed to air dry. Any pollen remaining on the slide was stained with one-two drops of Calbera's fluid[44] and a cover slip was applied to the surface of the drop. All pollen identified under light microscopy was compared to the pollen library. Washed insect specimens were stored in vials of 70% EtOH for transport. They were then dried, pinned, and sent to the Bishop Museum (Honolulu, HI) for identification. The lengths of the insects were measured prior to pinning (n = 15).

To determine the location of *G. tomentosum* pollen on beetle bodies while they foraged in flowers, an additional 36 beetles were removed from flowers, euthanized in jars of ethyl acetate fumes but were stored dry in paper tissues for transport. An Environmental Scanning Electron Microscope (ESEM) was used to determine where and how grains were attached to the beetle's exoskeleton. These specimens were attached to a stub, spatter coated in gold, and examined with an ESEM at low vacuum.

The ESEM was also used to count the number of grains on each beetle. To determine whether the beetle manipulated and ate pollen grains we examined their mouthparts under the ESEM to look for grains in the process of ingestion. We also dissected the GI (gastrointestinal) tract of an additional five beetles under a light microscope to look for ingested grains or grain fragments.

Experiments on floral foragers

Tracing experiments were used to determine what attracted nitidulid beetles to a *G. tomentosum* flower. Nitidulids were collected from both study sites and starved for 24 hours. Tracing experiments consisted of recording the search pattern of nitidulid beetles and the amount of time they spent prior to contacting the attractant (see protocols described by Acar et al).[45] A filter paper (9 cm diameter) was inserted into a petri dish and the attractant was placed at the center of the filter paper. Nine attractant sources were tested on beetles collected randomly from both study sites.

1. Droplets of sucrose at concentrations of 0.3 to 0.5 ppm.
2. Droplets of fructose at concentrations of 0.3 to 0.5 ppm.
3. Droplets of glucose at concentrations of 0.3 to 0.5 ppm.
4. *G. tomentosum* pollen
5. *G. tomentosum* petal piece
6. petal and sugar concentrate
7. yellow paper and sugar concentrate
8. yellow paper and pollen
9. yellow paper only (the yellow paper was the same color as the petals of *G. tomentosum* flowers)

Once the stimulus was in place a beetle, which had been starved for 24 hours, was released on the inside edge of the petri dish. The dish was covered and a piece of transparent paper was placed over the top. The path the beetle followed to the stimulus was traced on transparent paper, and the length of time it took the beetle to find the stimulus was recorded. If the beetle had not found the source within five minutes it was counted as not found. Ten beetles were used to test each stimulus source. After the experiments, the beetles were returned to the *G. tomentosum* populations.

Statistical analyses

To determine if there was a difference in fruit production between the two study sites, a Welch Modified Two-Sample *t*-test, without assuming equal variance, was used to compare the total fruit counts between the two research sites.[46]

The data were pooled for all plants for a total of 14 days and 142 plants (n = 142). The percentage of total fallen stigmas was calculated, and the mean percent and standard deviation calculated for the two populations together. A box-plot showed no need for a transformation of the data. Therefore, a standard

two-sample *t*-test, using S-plus, was performed on the data comparing the mean percentage of fallen stigmas on a *G. tomentosum* plant in the morning to the late afternoon.

To determine if the plants could self-pollinate as well as cross-pollinate, we performed a *t*-test,[47] assuming equal variance, comparing the Self vs. Cross percentage of pollen tubes that penetrated the ovary. The null hypothesis was that there would be no statistical difference between cross and self pollination treatments.

A fixed effects analysis of variance (AOV) with a Tukey post hoc test was performed to determine if there was a significant difference in pollen load between locations on the beetle.[46] A box-plot showed outliers and many zeros, therefore the data underwent a log (+1) transformation. The least squares means was used to detect significant differences between the head, thorax/abdomen, and terminal segment/genitalia groups.[46]

To determine if the beetles showed a visual preference in the tracing experiments, a logistic regression in SAS was used to compare all the treatment groups. The treatment groups were compared individually. No intracolour differences were present; therefore we collapsed the treatments as with and without color to increase our power for detecting differences between the means of the tracing results.

Results

Floral life-span, dimensions and fruit set

The *G. tomentosum* flowers in the two populations had a mean corolla span of 4.91 ± 0.82 cm, and a mean corolla height of 3.29 ± 0.45 cm. The base of the corolla to the tip of the stigma measured a mean of 4.1 ± 0.62 cm.

Flowers of *G. tomentosum* opened for only one day. The corolla began to expand around 0700 h and the petal lobes were fully expanded by 1130 h. Stamens and stigma matured simultaneously. Stigmas were receptive and most stamens dehisced shortly before the bud opened. Otherwise, some stamens didn't dehisce until 1–2 hours after the flower opened.

As the sun set around 2000 h the corolla began to collapse and became translucent at its margins. On the morning of the second day the wilted corolla remained closed but it persisted on the flower for several days prior to its abscission. The style fell off with the corolla but the calyx remained persistent around the ovary during fruit set. Of the 36 flowers tracked, 13 developed capsules with swollen ovaries visible within three to four days following the corolla wilt. Of those 13, only four reached maturity and the capsules dehisced exposing the tomentose seeds 25–30 days after initial blooming. By the end of 19 days, the other nine fruits were missing but we were unable to find evidence of fruit predation.

The average fruit set size at population I was 140.0 ± 37.4 fruits and 293.2 ± 90.9 at population II ($P = 0.013$). Total fruit counts through time for sites I and II indicated an increasing number of fruits maturing through June (Fig. 1). Fruiting peaks were around June 30th. The rise and decline in the fruit set at population II was probably due to dispersal as fruits inflated before they fell off the plant and were probably washed away by frequent down-slope rains. The contents of the three to four locules of dehiscent capsules were 8.65 ± 3.4 normal seeds and 1.5 ± 1.8 abortive seeds per fruit.

Compatibility system

Pollen tubes germinated and penetrated the stigma tissue entering the style within 24 hours regardless of whether the pistil was cross or self-pollinated (Figs. 2, A–C). There was no overt evidence of typical early or late-acting self-incompatibility responses in self-pollinated pistils such as pollen tubes with swollen tips, tubes penetrating the style but then turning and growing upward, heavily callosed tubes etc. However, pollen tubes penetrated the ovary more often and more rapidly in cross-pollinated vs. self-pollinated pistils (Table 1) over the same time period. The comparative rate of pistil penetration in self vs. cross treatments was significant ($P = 0.0171$).

Mechanical self-pollination

A small percentage of the flowers on all 17 plants showed a form of mechanical self-pollination in which the style collapsed and the stigma bent over and touched the dehiscent stamens on the sheath surrounding the style. Out of 142 flowers tracked we found a ratio of 0.072 ± 0.052 with bent over stigmas (Fig. 3). Tracked flowers with bent stigmas were first noted as early as one hour after corolla expansion but some did

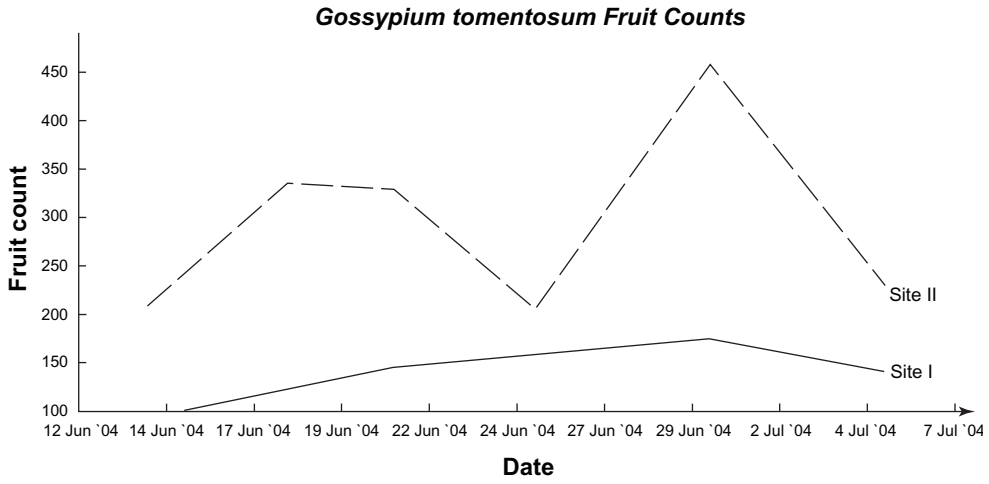

Figure 1. Comparison of total number of fruits produced during flowering season between *Gossypium tomentosum* populations.

not start to bend until after 1200 h. We recorded rates of bent stigmas in the morning (from 700–1100 h) and the afternoon (from 1400–1700 h). The null hypothesis was rejected because rates of stigma bending failed to increase as the flower aged. When data from both populations was pooled over a total of 14 days and (n = 142 flowers tracked) 0.048 ± 0.44 of flowers had bent stigmas in the morning while 0.87 ± 0.049 had bent stigmas by late afternoon or dusk. There was no statistical difference between AM and PM rates of bending ($P = 0.09$).

Analyses of attractants and rewards

We did not observe darkened central patches at the base of the flower or contrasting, central blotches indicative of beetle-pollinated flowers in other parts of the world.[44,48] We were unable to detect a floral fragrance after removing the lid of scent jars two hours after flowers of *G. tomentosum* were placed inside. The flowers did not produce nectar. Clear liquid was observed and collected in the floral cup on only three occasions and the Brix reading was 0.00 in all three cases. Some flowers must have retained water after regular rains.

Floral foragers

The majority of visitors observed and collected on *G. tomentosum* were identified as *A. concolor*. The average length of the nitidulids was 3.2 mm. Human observations did not interfere with beetle behavior. Beetle visits to flowers began at 0700 h and the insects vacated the closing flowers after 1900 h (Fig. 4). Most copulating pairs of *A. concolor* were found in between the petals. Otherwise, beetles appeared to feed almost continuously on the anthers with brief lulls in foraging activity between 1130 h and 1330 h when many were found resting on petals (Fig. 4). *Aethina concolor* usually entered the flower by landing on the

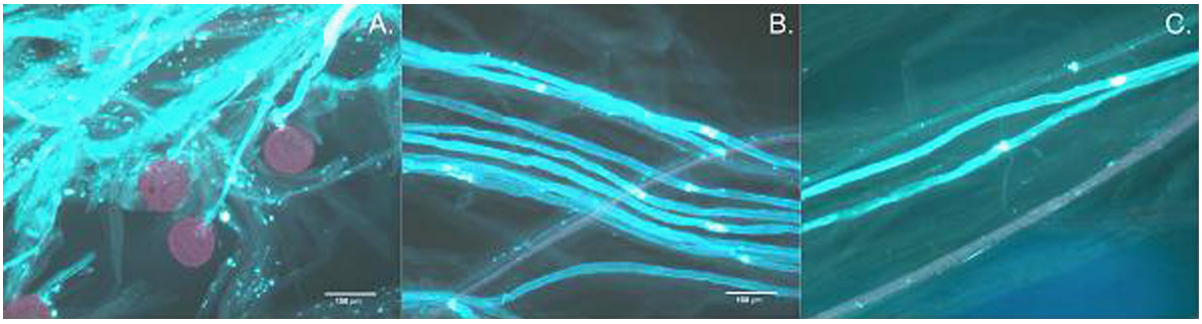

Figure 2. Fluorescent Micrographs of *Gossypium tomentosum* pollen and pollen tubes: **A**) *G. tomentosum* pollen on the stigma of the flower, **B**) Pollen tubes in style of *G. tomentosum* in the Cross pollination treatment, **C**) Pollen tubes in style of *G. tomentosum* in the Self-pollination treatment.

Table 1. Comparative rates of pistil penetration in hand pollination studies.

Treatment	n (number of flowers)	Number of pollen grains on stigma	Number of pollen tubes reaching ovary	Percent of pollen tubes to reach plant ovary
Self	9	96.8 (±80.7)	6.3 (±5.2)	7.5 (±3.86)
Cross	9	107 (±33.4)	12.8 (±5.6)	11.9 (±3.1)

Notes: Comparative number, mean (±SD), of pollen on stigma lobes and growth of pollen tubes in *Gossypium tomentosum* pistils in the hand pollination studies ($P = 0.017$).

petals and then crawling up the staminal tube sheathing the style. They exited the flower by crawling up onto the pollen-receptive surface of the stigma and then they launched themselves into the air. Additional field observations showed that *A. concolor* was the only visiting insect to contact the stigma frequently and regularly (Table 2).

Pollen load analysis showed that *A. concolor* was more likely to carry the pollen of *G. tomentosum* pollen compared to any other insect visitors to the flower (Table 3). A single honeybee (*Apis mellifera*) visited *G. tomentosum,* contacted the stigma, and was found to carry mixed loads of pollen. Other insects carrying grains of *G. tomentosum* were not observed to contact the stigma during their visits.

Grains of *G. tomentosum* were usually clumped on one to three sites on each beetle observed under SEM. This included the head, thorax and abdomen and specifically on the terminal segment of the abdomen bearing the beetle's genitalia. The mean number of pollen grains on the head was 3.78 ± 5.04. There were 5.4 ± 3.98 grains distributed on the combined thorax and abdomen. There were 1.76 ± 3.3 grains on the terminal segment. Comparative deposition of pollen on the beetle's body was statistically significant on the combined thorax and abdomen vs. the terminal segment ($P = 0.0003$) and the terminal segment vs. the head ($P = 0.0389$) (Fig. 5). Exine spines on the tectate pollen wall became entangled in the setae on the beetle's exoskeleton (Figs. 6, A-B).

Pollen grains were found in the mouth parts of the beetles (Fig. 6, C) and in the GI tract (Fig. 6, D). The pollen grain wall in the GI tract was whole, but deflated, suggesting that this beetle has a digestive physiology (similar to bees) in which hydrated grains rupture inside the GI releasing their cytoplasmic contents.

Experiments on floral foragers

Tracing experiments of beetles in petri dishes indicated (Table 4) that the beetles were more likely to visit yellow models and pollen of *G. tomentosum*. When the treatment groups without color were collapsed and compared to those treatments with color, the *P*-value approached significance ($P = 0.054$).

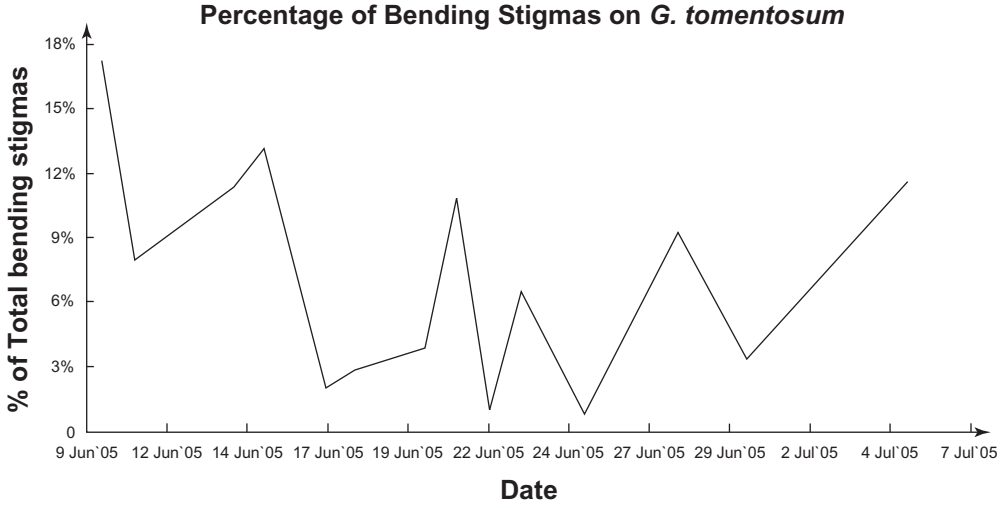

Figure 3. Percentage of total flowers on *Gossypium tomentosum* plants that have bending stigmas, monitored through growing season.

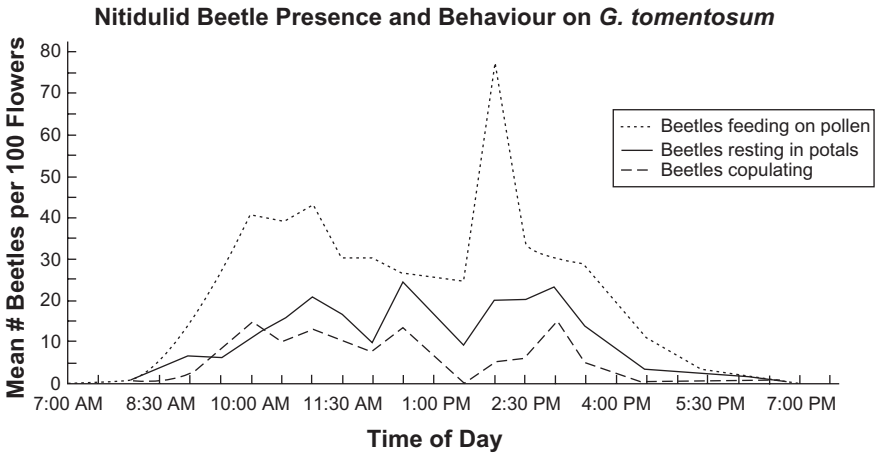

Figure 4. *Aethina concolor* behavior and presence on *Gossypium tomentosum* plotted over time. Beetle behavior is separated into beetles resting in petals, copulating, and feeding on pollen.

Discussion

A study by DeJoode and Wendel[36] concluded that *G. tomentosum* had relatively limited genetic diversity paralleling a similar conclusion based on morphological evidence by Stephens.[38] Our results indicate, though, that our populations of *G. tomentosum* were not obligate in-breeders. While self-pollination is an important "failsafe mechanism" in this species it probably benefits from some out-crossing. Our results showed that maturation and survival from pollinated pistil to dehiscent capsule with viable seed set was low in both populations. Many ovules within the same locules failed to complete development.

More important, fluorescence analysis showed that pollen tubes produced by a hand-manipulated cross-pollination reached the ovary faster than tubes generated by self-pollination. This parallels systems in other threatened or endangered angiosperms showing

Table 2. Observations of insect visitors contact with *Gossypium tomentosum* stigmas.

Date	Time	Location	Visitor
5-Jun-04	8:30	Plant 3	*A. concolor*
9-Jun-04	15:05	Plant 7	*Apis mellifera*
10-Jun-04	9:27	Plant 7	*Apis mellifera*
10-Jun-04	9:30	Plant 5	*A. concolor*
10-Jun-04	9:40	Plant 5	*A. concolor*
10-Jun-04	10:30	Plant 7	*A. concolor*
10-Jun-04	10:45	Plant 7	*A. concolor*
10-Jun-04	11:45	Plant 6	*Apis mellifera*
11-Jun-04	11:00	Plant 10	*A. concolor*
11-Jun-04	11:15	Plant 17	*A. concolor*
11-Jun-04	11:20	Plant 17	*A. concolor*
14-Jun-04	10:10	Plant 7	*Linepithema humilis*
18-Jun-04	9:30	Plant 8	*A. concolor*
18-Jun-04	10:30	Plant 3	*A. concolor*
21-Jun-04	11:00	Plant 17	*A. concolor*
22-Jun-04	11:00	Plant 3	*Toxomerus marginatus*
23-Jun-04	15:00	Plant 17	*Ceratina nr. dentipes*
23-Jun-04	3:15	Plant 10	*A. concolor*
30-Jun-04	10:15	Plant 6	*A. concolor*
l-Jul-04	10:45	Plant 7	*Apis mellifera*
l-Jul-04	11:15	Plant 3	*A. concolor*
l-Jul-04	11:17	Plant 3	*A. concolor*
5-Jul-04	10:00	Plant 6	*A. concolor*

Table 3. Pollen grains removed from the insect visitors of *Gossypium tomentosum*.

Insect taxa	n	Average pollen load from *Gossypium*	Average pollen load of other than *Gossypium* pollen
Aethina concolor	20	19.75	0.35
Apis mellifera	1	14.00	12.00
Ceratina nr. dentipes	4	11.00	7.25
Grasshopper*	1	0.00	0.00
Hyalopeplus pellucidus	2	0.00	2.00
Linepithema humilis	3	0.33	0.00
Pieris rapae	1	0.00	2.00
Toxomerus marginatus	2	9.50	18.5

Notes: The grasshopper specimen was damaged during shipping and could not be identified.

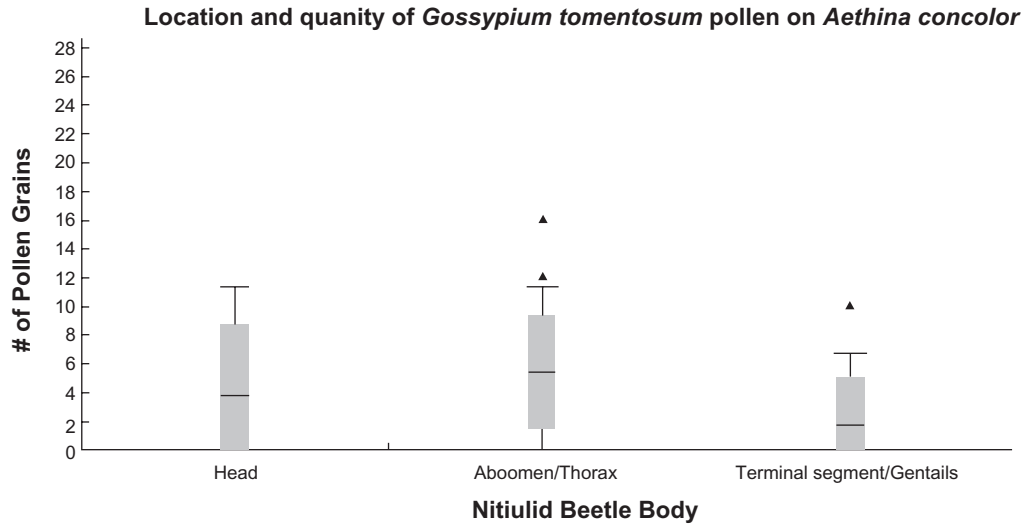

Figure 5. Location and quantity of *Gossypium tomentosum* pollen load carried by *Aethina concolor* beetles. Comparative deposition of pollen on the beetle's body was statistically significant on the combined thorax and abdomen vs. the terminal segment ($P = 0.0003$) and the terminal segment vs. the head ($P = 0.0389$).

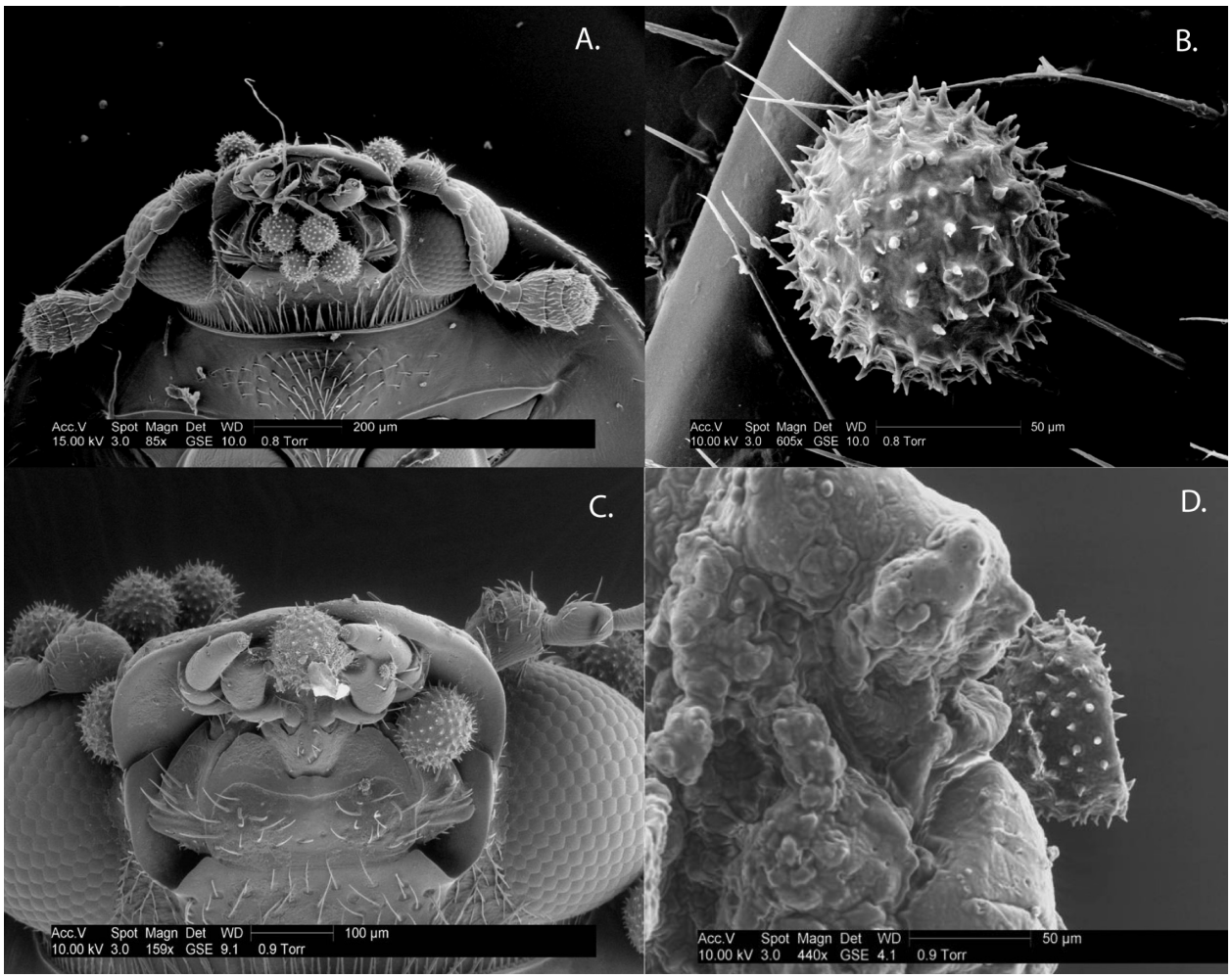

Figure 6. ESEM Micrographs **A**) *Aethina concolor* with *Gossypium tomentosum* pollen clustered on the head. **B**) *Gossypium. tomentosum* pollen in the setae of a nitidulid beetle, *Aethina concolor*. **C**) *Aethina concolor* with *G. tomentosum* pollen in its mandibles. **D**) *Gossypium. tomentosum* pollen inside the dissected GI tract of a nitidulid beetle.

Table 4. *Aethina concolor* that located treatment source.

Treatment	n	Percentage of beetles that found source
Gossypium pollen	22	13.64%
Gossypium petal	21	28.57%
Yellow paper	15	46.67%
Glucose (20 µl)	5	20.00%
Glucose (10 µl)	10	10.00%
Sucrose (20 µl)	5	0.00%
Sucrose (10 µl)	9	33.33%
Fructose (20 µl)	4	0.00%
Fructose (10 µl)	10	10.00%

some inbreeding depression based on lower levels of pollinator-mediated cross-pollination.[4]

When self-pollination evolves due, presumably, to low frequencies of cross-pollination[5,49,50] both mechanisms promoting cross and self-pollination may continue to coexist in the same flower. This is often referred to as a delayed selfing mechanism. This can happen, as in this study, with preferential pollen tube growth rates[27,51] in which xenogamous pollen tubes grow faster than the autogamous ones. While pollen tubes based on cross-pollination reach the ovary first, tubes based on self-pollination eventually reach the ovules as well effecting seed set when xenogamous depositions of pollen are low or non-existent.

A second mechanism for delayed self-pollination occurs through morphological developments as the flower ages and anthers and stigmas contact each other when pollen vectors fail to arrive.[49,50,52] This mode of contact is usually associated with a proscribed period of temporal delay. Self-pollination occurs when the anthers collapse onto the flower's own stigma at the end of the day, or just prior to the conclusion of stigmatic receptivity() as described in *Kalmia latifolia*[53] and *Sanguinaria canadensis*.[54] Stigmas, or stigma lobes, bending into dehiscent anthers have been recorded in other members of Malvaceae including *Hibiscus trionum*[50] and *Cienfuegosia argentina*[39] but delayed, mechanical self-pollination is not unique to the Malvaceae. In other populations of unrelated species, subspecies or biotypes self-pollination occurs predictably in all flowers on the plant as each flower ages in the absence of pollen vectors. This is particularly common in some plants families including the Orchidaceae,[55] Nymphaeaceae[56,57] and in a number of temperate herbs associated with seasonally stressed flowering periods within alpine zones, ruderal habitats and shady forest floors.[27,54]

However, our results showed that, while mechanical autogamy occurred in two small populations of *G. tomentosum*, it remained only a partial trend within each genet. A mean of only 7% of the chasmogamous flowers in the pooled populations bent their receptive stigmas into their dehiscent anthers. Mechanical self-pollination in these few flowers species was not consistent with the usual floral processes that occur as a result of ontogenetic aging. The incomplete trend towards mechanical self-pollination and differential pollen tube growth in *G. tomentosum* indicates that vector-mediated pollination (self- and/or cross) remains of some importance, at least in the parent population(s) that produced the current 17 genets surviving within the National Tropical Botanical Garden. Insect-mediated self-pollination, and perhaps some insect-mediated cross-pollination, probably produced the majority of fertile capsules in the Kauai populations.

What were the original pollinators of these showy, yellow flowers? *G. tomentosum* is phylogenetically nested within a clade with four other *Gossypium* species that do produce nectar.[58] Therefore, making floral nectar is a likely ancestral trait, with *G. tomentosum* having lost this ability. Crepuscular pollen vectors now seem unlikely considering the diurnal life-span of the flowers and the total absence of floral nectar. One is more likely to predict bee-pollination based on floral presentation, the pollen reward and the absence of floral nectar.[52,59] However, we still have no evidence that any of the endangered or extinct native *Hylaeus* (*Nesoprosopis*, Colletidae) spp. ever visit or visited extant populations of *G. tomentosum*.[60] It is interesting to note that hedges of the yellow-flowered, Polynesian, *Hibiscus tiliaceus* (Malvaceae) bloomed extensively within a few meters of one of the populations of *G. tomentosum*. Bernhardt (unpublished) frequently observed the naturalized, *Xylocopa sonorina* visiting flowers of *H. tilaceus* but this polylectic bee ignored *G. tomentosum* at least within the grounds of the National Tropical Botanical Garden.

It may be more reasonable to assume that the original pollinators of *G. tomentosum* were beetles, particularly anthophilous members of the Scarabaeidae. Members of this family are common pollinators of tropical

angiosperms in several families.[63] *G. tomentosum* may have exploited a native pollen-eating beetle that also climbed up the style and departed via the receptive stigma.

Beetle pollination predates angiosperms and was probably part of early gymnosperm pollination systems.[27,61,62] While the identity of nitidulid fossils in Mesozoic rock remains uncertain, Cretaceous amber does contain specimens identified to the family, Nitidulidae[63] which suggests that some nitidulids existed concurrently with the ancestors of some basal angiosperm lineages. In general, anthophilous beetles may visit a flower for a number of rewards including nectar, pollen, starchy food bodies, warmth, concealment and prospective mates.[3,61] Beetle pollinators often bear mouthparts adapted specifically for harvesting and consuming pollen and they may be attracted specifically to canalized color and/or scent patterns.[61,64]

Nitidulid beetles can be an important selective pressure on plants. For example, genera such as *Guatteria* are adapted to nitidulid pollinators.[23] However, in Hawaii, while *G. tomentosum* is an endemic species, the nitidulid *A. concolor* is not. This, however, does not rule out the possibility that *G. tomentosum* was originally selected for beetle pollination in its evolutionary history. In this case, an invasive species may be enhancing the survival of an endemic species. Some nitidulids are endemic to the Hawaiian Islands; about 177 nitidulid species have been described on 35 different plant families.[40]

This study sought to determine the benefit the beetle receives from visiting the plant. Nitidulids traditionally feed on decaying fruit or nectar, which is why they are called "sap beetles". However, *G. tomentosum* produces no nectar or fleshy fruit,[11] as was verified for these populations. The data clearly show that the source of the nitidulid's nutrition was the *G. tomentosum* pollen. The beetles were observed feeding on the stamens, and were also observed eating pollen in a petri dish in the lab. The nitidulids move the whole pollen grain into their mouths with their two front legs, even reaching up with their front legs to sweep the pollen off their heads and into their mouths. The ESEM results (Fig. 6C) show whole pollen grains in the mouth parts of the nitidulid, which means the spiny pollen is easily consumed. Pollen grains found in the dissected gut of the nitidulid (Fig. 6D) verify the consumption of pollen by the beetle. No bite marks were ever found on *G. tomentosum* petals, and our data suggest that pollen is the main nutrition source in the beetle's diet.

The nitidulids also benefit by hiding in the petals of the *G. tomentosum* for refuge and mating purposes. However, the beetles did not remain in the plants overnight, a characteristic common in many basal angiosperms pollinated exclusively by beetles.[61] No beetles were ever found on a *G. tomentosum* plant before 0800 h or after 1900 h. Although further study is needed to understand the behavior of the nitidulid beetles, this time scale is important in identifying them as consistent pollinators.

Previous research on the attraction of nitidulid pollinators is focused mainly in the palm family (Arecaceae) and show a variety of plant rewards offered, including scent, thermogenesis, and visual stimulation[30,65] that nitidulids respond to.

In contrast, *Gossypium tomentosum* did not produce any discernible scents. Instead, our tracing experiments found that a simple, yellow, visual cue was most likely to attract *A. concolor*, even when the cue was a piece of paper instead of a yellow petal (Fig. 7). However, experiments showed that *A. concolor* was also attracted to pollen of *G. tomentosum* and the lipophilous coat on a pollen grain wall is often a source of volatiles.[64]

A. concolor benefits from *G. tomentosum*, but does a true mutualistic relationship exist? Because nitidulids are small, it has been questioned whether or not they are primary pollinators effecting a selective force on the reproductive fitness of the plant. These small beetles are usually overlooked, or merely listed as a secondary or an incidental presence on a plant, and rarely included in the floral biology of a species. For example, while Liu et al[29] listed nitidulids on a chart as possible pollinators of members of the family, Calycanthacaeae family, there were not recorded as primary pollinators.

However, some research shows that various species of nitidulid beetles are recognized as primary pollinators. *Meligethes aenus* pollinates *Narcissus angustifolius*.[16] Nitidulids are primary pollinators of members of the palm family[19] including, the babassu palm, *Orbignya phalerata*,[17] and several species in the genus *Annona*.[18,21,67,66] The association between nitidulids and palms is determined to have a long co-evolutionary history[18,21,65,68] in which the nitidulids are

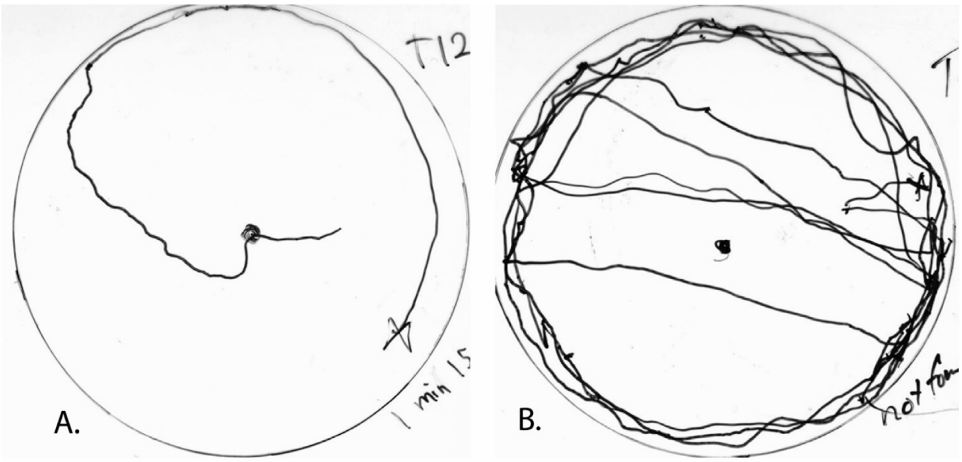

Figure 7. A sample of tracing patterns on the transparencies from the tracing experiments with *Aethina concolor* beetles A. Beetle tracing pattern when treatment source had a color component. B. Beetle tracing pattern when treatment source had no color component.

feeding on nectar and pollen, while the fruit set of the plants is increased when nitidulids are present.

There are several factors that create uncertainty about the role of *A. concolor* as primary pollinators of *G. tomentosum*. Within this study, these concerns included the size and foraging range of the nitidulid, and whether or not stigma-beetle contact was consistent.

The first concern is whether or not the nitidulids are large enough to carry pollen and if they have a foraging range that is large enough to be a pollen dispersal vector.[32,69] Small beetles are considered to have a small foraging range.[27] While they eat pollen and carry it readily they do not always transport grains from plant to plant or from population to population. However, one SEM study[24] found small beetles to be important carriers of pollen. Our ESEM study clearly shows pollen clinging to the beetle in copious amounts (Figs. 6, A-B). The ESEM pollen load analysis reveals that the Thorax/Abdomen of the beetle carries the majority of the pollen, deposited within the beetle's setae (Figs. 5, 6,D).

The second concern is whether stigma-beetle contact occurred with regularity. It is the act of exiting the flower that the beetle is most likely to contact the stigma of the *G. tomentosum*. Beetles usually take off from the highest point they can crawl to, both as an energy saving mechanism, and in order to achieve the necessary lift to fly.[64] Even if *A. conolor* do not have a large foraging range, and may even spend all day in the same flower, this flight behavior and the timing of beetle presence in *G. tomentosum* means that there is at least one time of the day when stigma-beetle contact is regular. As pollen load analysis showed that only *G. tomentosum* pollen was present on *A. concolor*, it suggests that nitidulids have a small and specialized foraging range within a large and floriferous botanical garden. Our results indicate that as stigma contact by the beetle occurs at many different times of the day which suggests that a nitidulid may exit more than one flower each effecting some geitonogamous or even xenogamous pollination. Further testing of nitidulid range and tracking of the number of flowers visited by a single beetle is required before we can conclude that nitidulids are responsible for regular pollen transfer between flowers.

This pollination by nitidulid beetles appears to increase the reproductive fitness of *G. tomentosum.* Increased self-pollination can be one of the detrimental effects of exotic pollinators on native plant species.[8] However, *G. tomentosum* is self-compatible[70] and the elevated stigma in relation to the lower stamens appears to lower opportunities for mechanical self-pollination. The nitidulid beetles probably provide some cross-pollination, and definitely enhance rates of self-pollination.[70]

In conclusion, we conclude that the naturalized nitidulid beetle, *A. concolor*, is now the primary pollinator of *G. tomentosum* in the National Tropical Botanic Garden on Kauai. Plant and beetle enjoy a standard mutualistic relationship with the insect deriving nutrition, shelter, and a mating site, all standard benefits of beetle-pollinated flowers.[61] For both populations of *G. tomentosum*, the nitidulid is now

their only known pollen vector for enhanced self- and possibly cross-pollination increasing reproductive fitness. At least two pollination systems persist in *G. tomentosum*. There is entomophily via nitidulid beetles and some degree of mechanical autogamy.[70]

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Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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