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Source: Florida Entomologist, 102(1) : 154-160

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.102.0125

Aspects of the pollination biology of *Encyclia tampensis*, the commercially exploited butterfly orchid, and *Prosthechea cochleata*, the endangered clamshell orchid, in south Florida

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Abstract

Encyclia tampensis (Lindl.) Small (Orchidaceae), the butterfly orchid, is a commercially exploited, epiphytic orchid native to Florida. Similarly, *Prosthechea cochleata* (L.) W.E. Higgins (Orchidaceae), the clamshell orchid, is an endangered orchid that is epiphytic and native to Florida. We conducted this study in southern Florida at the Florida Panther National Wildlife Refuge to gain more information about the pollination biology of *E. tampensis* and *P. cochleata* (var. triandra). Experiments using pollinator exclusion bags revealed that *E. tampensis* is not capable of spontaneous self-pollination, and requires a pollen vector for seed capsule development; however, *P. cochleata* appears to be readily self-pollinating. Using active and passive sampling, we determined that *E. tampensis* potentially can be pollinated by a variety of flower-visiting insects, including Hymenoptera, Diptera, and Coleoptera. Insects from all 3 orders were observed on and collected from the *E. tampensis* flowers. However, only insects from 1 order (Hymenoptera) were actively collected from *P. cochleata.* Our data are useful for conservation efforts for *E. tampensis* and *P. cochleata*, because knowledge about potential pollinators and self-pollination capability can lead to future studies and information about optimal habitats for outplanting and reintroduction. The orchids face decline due to habitat loss, pests, and poaching, so conservation is an important key to re-establishment of these species.

Key Words: Orchidaceae; pollinators; netting; traps

Resumen

Encyclia tampensis (Lindl.) Small (Orchidaceae), la orquídea mariposa, es una orquídea epífita y nativa de la Florida, que es comercialmente explotada. Del mismo modo, *Prosthechea cochleata* (L.) W.E. Higgins (Orchidaceae), la orquídea de la concha de almeja, es una orquídea en peligro de extinción que es epífita y nativa de la Florida. Se realizó este estudio en el sur de Florida en el Refugio Nacional de Vida Silvestre Panther de Florida para obtener más información sobre la biología de polinización de *E. tampensis* y *P. cochleata* (var. triandra). Los experimentos con bolsas de exclusión de polinizadores revelaron que *E. tampensis* no es capaz de autopolinizarse espontáneamente y requiere un vector de polen para el desarrollo de la cápsula de la semilla, sin embargo, parece que *P. cochleata* se autopoliniza fácilmente. Mediante el uso de muestreo activo y pasivo, determinamos que *E. tampensis* puede ser polinizada por una variedad de insectos que visitan las flores, como himenópteros, dípteros y coleópteros. Los insectos de los tres órdenes fueron observados y recolectado de las flores de *E. tampensis*. Mientras tanto, solo insectos de un orden (Hymenoptera) fueron recolectados activamente de *P. cochleata*. Nuestros datos son útiles para los esfuerzos de conservación de *E. tampensis* y *P. cochleata*, ya que el conocimiento sobre los posibles polinizadores y la capacidad de autopolinización pueden conducir a estudios futuros e información sobre hábitats óptimos para la plantación y la reintroducción. Las orquídeas enfrentan un declive debido a la pérdida de hábitat, plagas y caza furtiva, por lo que la conservación es una clave importante para el restablecimiento de estas especies.

Palabras Clave: Orchidaceae; polinizadores; redes; trampas

Members of the flower family Orchidaceae, the orchids, are known to be pollinated by a diverse set of taxa. The most common orchid pollinators are bees and wasps in the order Hymenoptera. However, insects in Lepidoptera, Diptera, Coleoptera, and other orders are known orchid pollinators as well (Statman-Weil 2001; Lehnebach & Robertson 2004; Micheneau et al. 2010; Stökl et al. 2011). Orchids have various relationships with pollinators. Some species are pollinated by multiple pollinator species, whereas others are pollinated by only a single species. Orchid flowers have the same basic floral structures for pollination across most species.

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There is a central structure referred to as the column that contains both the male (anther) and the female (stigma) parts of the flower (Roberts & Dixon 2008). Below the column, a petal that has been modified into a labellum or lip acts as a landing area for pollinators. This directs pollinators to the nectar source within the flower, causing them to contact the orchid pollen (Brown 2005). Unlike loose pollen grains produced by most flowering plant families, orchid pollen is housed in compact structures called pollinia. Pollen transfer occurs when the pollinia are attached to a visiting insect and transferred between flowers. When pollination occurs, the ovary will begin to swell and form a seed capsule filled with millions of seeds (Roberts & Dixon 2008).

Florida is home to over 100 species of orchids. Most occur in the southernmost areas of the state, where these locations provide ideal growing conditions for many epiphytic species (Brown 2005). Several protected parks and refuges in south Florida provide habitat for these species, over half of which are listed as threatened or endangered (Stewart & Richardson 2008). Despite these protected areas, many orchids still face threats from habitat degradation, invasive species competition, poaching of plants, and pests. In a survey of orchid pests in southern Florida, *Encyclia tampensis* (Lindl.) Small (Orchidaceae), the butterfly orchid, and *Prosthechea cochleata* (L.) W.E. Higgins (Orchidaceae), the clamshell orchid, were found to have Boisduval scale (*Diaspis boisduvalii* Signoret) (Diaspididae) present on some of the adult plants (Ray et al. 2012; Zettler et al. 2012).

Currently, there is little information regarding pollination of the Florida butterfly orchid (Fig. 1), which is listed as commercially exploited, or the clamshell orchid (Fig. 2), listed as endangered on Florida's Regulated Plant Index. A better understanding of these orchids' pollinator(s) will facilitate science-based future conservation decisions for these threatened species.

The objectives of this study were to identify the potential pollinator(s) of *E. tampensis*, compare seed capsule formation between flower locations, and identify factors that influence differences in seed capsule formation. Furthermore, we sampled for potential pollinators of *P. cochleata* to compare any collected insect taxa between the 2 orchid species.

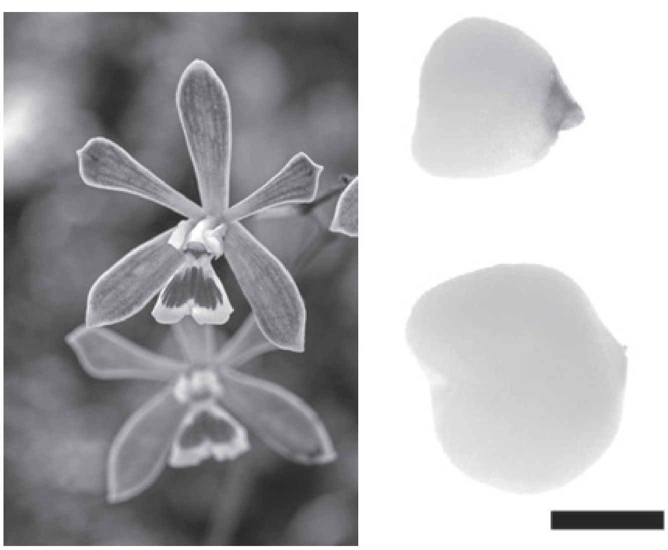

Fig. 1. Left: *Encyclia tampensis*, the Florida butterfly orchid, growing at the Florida Panther National Wildlife Refuge in Collier County, Florida. Photograph by Larry W. Richardson. Right: Two orchid pollinia with 1 mm scale bar. Photograph by Lawrence E. Reeves.

Fig. 2. Flowers of *Prosthechea cochleata* on an orchid growing in the Florida Panther National Wildlife Refuge, in Collier County, Florida. Photograph by Larry W. Richardson.

Materials and Methods

POLLINATOR EXCLUSION

This study was conducted at the Florida Panther National Wildlife Refuge in Collier County, Florida, USA (26.171577°E, 81.347108°N), during 3 blooming seasons (2015–2017). Four separate locations at the refuge were selected (Fig. 3), designated as Locations 1 to 4. Each location had 3 sites, spaced 10 to 30 m apart, where *E. tampensis* orchids occur naturally. The GPS coordinates of these locations have been withheld due to the threatened status of these orchids and others growing in the area. Location 1 was a developed, landscaped work center at Florida Panther National Wildlife Refuge, whereas Locations 2 to 4 were all natural, freshwater wetland forest habitat. Each site had at least 3 blooming *E. tampensis*, with a total of 5 or more flowers per site. The number of flowers blooming at each site was recorded. To determine if the orchid requires a pollinator for seed capsule production, mesh exclusion bags made by the authors (approximate measurements: 125 mm length × 90 mm width, 5 holes per 1 mm of fabric) were placed over at least 3 unopened or newly opened, unpollinated flowers at each location (Fig. 4). When exclusion bags were placed over a flower, they typically were covering an inflorescence of multiple flow-

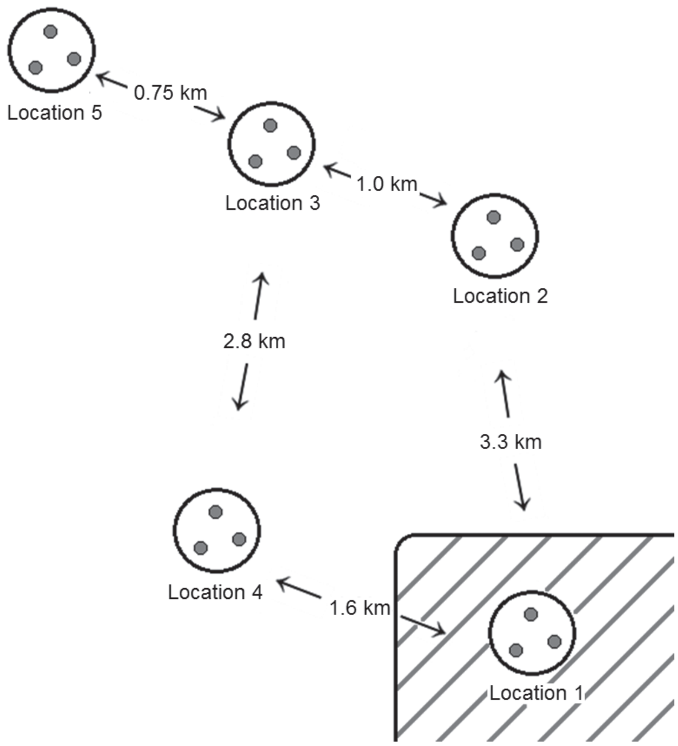

Fig. 3. Map of the locations at the Florida Panther National Wildlife Refuge from which *Encyclia tampensis* and *Prosthechea cochleata* flowers were sampled during the 3-yr study (flowers of *E. tampensis*: Locations 1 to 4; *P. cochleata*: Locations 1, 3, 5). Location 1 is the developed site, and locations 2 to 5 are natural swamp habitat. Three replicates were used in each location. The GPS coordinates of these locations have been withheld due to the threatened status of these orchids and others growing in the area.

Fig. 4. A fine mesh exclusion bag placed around 2 *Encyclia tampensis* flowers that had opened soon after being covered. Exclusion bags were used to determine if insect pollinators were important to seed capsule production. These bags prevented any potential pollinators from visiting the flowers. Photograph by Haleigh A. Ray.

ers, not a single flower per bag. Evidence of previous pollination was determined by visually inspecting the flower stem. There is a noticeable difference in pollinated flowers, because seed capsule production begins quickly after fertilization (Fig. 5). The flower stems change color from a yellow color when unfertilized to a dark green when fertilized, and begin to swell as the seed capsule develops.

POLLINATOR COLLECTION

During the study, potential pollinators were collected using traps, as well as by active sampling. Three types of traps were used to collect insects: blue vane traps (SpringStar®, Seattle, Washington, USA), WHY (wasp, hornet, yellowjacket) traps (Rescue®, Spokane, Washington), and colored insect bowl traps comprised of 3 painted bowls (blue, yellow, and white) (Blue Sky®, Brooklyn, New York, USA) (Fig. 6). Three traps of each type were set up at each location within 5 m of flowering *E. tampensis* or *P. cochleata* orchids. The blue vane and WHY traps were suspended from branches at approximately 1.5 m above the ground, whereas the bowl traps were placed at ground level. Though the orchids are epiphytic and can grow lower or much higher, 1.5 m was chosen to allow traps to be hung and checked easily. Each trap contained approximately 200 mL of water with 0.01% of the surfactant Silwet L-77 (Helena®, Collierville, Tennessee, USA) to break the surface tension and prevent insects from escaping. Traps were checked daily and insects were collected over a 6-d period between 1:00 PM and 4:00 PM during the peak blooming period each yr. The traps were available to insects 24 h per d. Insects collected from the bowls were pooled into 1 sample.

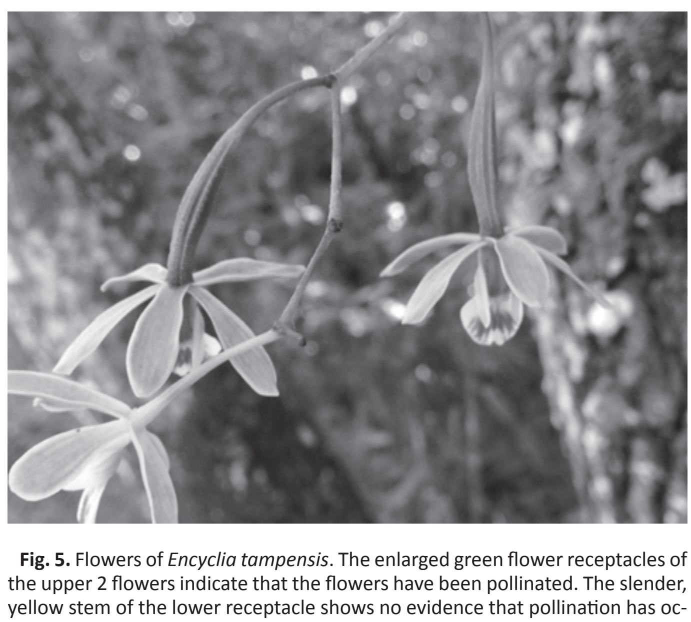

Fig. 5. Flowers of *Encyclia tampensis*. The enlarged green flower receptacles of the upper 2 flowers indicate that the flowers have been pollinated. The slender, yellow stem of the lower receptacle shows no evidence that pollination has occurred. Photograph by Haleigh A. Ray.

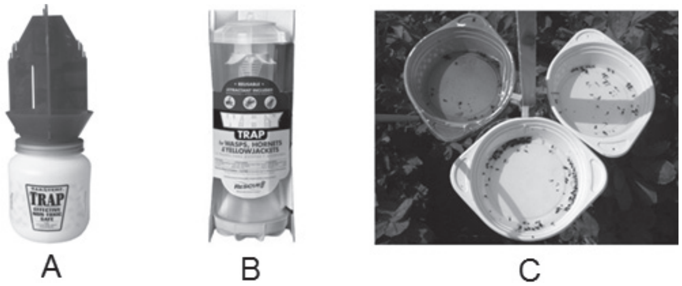

Fig. 6. Three types of insect traps were used to sample potential pollinators of *Encyclia tampensis*, the Florida butterfly orchid, and *Prosthechea cochleata*, the clamshell orchid. Blue vane traps (A), WHY (wasp, hornet, yellowjacket) traps (B), and painted insect bowl traps (C).

In addition to trapping, all locations were actively sampled twice each d in 45-min increments (15 min at each site) for a total of 90 min of observation at each location per d. When actively collecting insects, the locations were visited in varying order so that they were being monitored twice daily, once in the morning and once in the afternoon (e.g., day 1: location 1, 2, 3, 4; day 2: location 2, 3, 4, 1). This was to randomize locations visited during every collection period. Insects that landed on the floral blooms were collected using an aerial net. Collected specimens were identified and released, if possible, if a voucher specimen had been collected already, or preserved by freezing and taken to the Entomology and Nematology Department at the University of Florida for identification. All flowering plants within a 10 m radius from the orchids were photographed and identified. This study was repeated over 3 yr when the flowers were blooming, mid-May to early Jun from 2015 to 2017 for *E. tampensis*, and from Sep to Oct in 2015 to 2016 for *P. cochleata*. The *E. tampensis* flowers were found at Locations 1 to 4, and *P. cochleata* were blooming at Locations 1, 3, and 5.

STATISTICAL METHODS

In order to detect any significant differences between abundance of pollinators at each collection site, and also to determine differences in seed capsule formation, we used a 1-way analysis of variance (ANOVA). A *t*-test was performed to test for any difference in pollinator abundance in the morning collections compared to the afternoon collections. These statistical tests were completed using JMP Statistical Analysis Software (SAS Institute, Cary, North Carolina, USA).

Results

POLLINATOR EXCLUSION

During the 3-yr study period, the average total numbers of flowers ± SD at each location were 125 ± 35, 97 ± 17, 50 ± 3, and 53 ± 5 at Locations 1, 2, 3, and 4, respectively. Each location included 3 sites that were sampled each of the 3 yr (N = 36 total, or 4 locations × 3 sites per location × 3 yr) over the course of the study. During the 3 yr of the study, a total of 231 flowers (about 24% of all flowers) were covered with mesh exclusion bags across all locations at Florida Panther National Wildlife Refuge. Of these, no seed capsules formed from bagged flowers, making it unlikely that these flowers are capable of spontaneous self-pollination. Of the flowers that were left uncovered, the flowers at the developed location (location 1) produced a significantly higher percentage of seed capsules ($P < 0.0001$; F Ratio = 30.59; df = 11) (Table 1).

Table 1. Three-yr mean percentage of seed capsule formation (± SD) for flowers at each of the locations where *Encyclia tampensis* was studied at the Florida Panther National Wildlife Refuge. Location 1 was at a developed work center, whereas the other locations were in the natural refuge habitat. Row means with the same letter are not different at $\alpha \leq 0.05$.

Year	Location 1	Location 2	Location 3	Location 4
2015	25.6% ± 1.8 a	10.3% ± 2.3 b	8.8% ± 3.5 b	3.2% ± 2.9 b
2016	28.5% ± 7.3 a	14.3% ± 2.2 b	9.3% ± 8.5 b	8.2% ± 2.6 b
2017	21.2% ± 9.5 b	16.8% ± 1.5 b	5.8% ± 5.0 b	5.5% ± 0.8 b

POLLINATOR COLLECTION – *ENCYCLIA TAMPENSIS*

Over the 3 yr collection period, a total of 46 insects were captured by active sampling, and 83 were captured in the 3 different traps. The number of mosquitoes or non-insect arthropods collected in the traps was not included in our data due to the unlikelihood that they would be pollinators of this species. However, specimens were still checked for visible signs of pollinia before being discarded (Fig. 1), because mosquitoes have been recorded as pollinators of other orchid species (Statman-Weil 2001). The insects collected by active sampling consisted of 3 orders: Hymenoptera, Diptera, and Coleoptera, with Hymenoptera being the principal order collected. This was true for each of the 4 locations, with Hymenoptera being the most common, followed by Diptera and Coleoptera, respectively. Insects that were repeatedly net-collected from the orchid flowers over the course of the study were the delta flower scarab, *Trigonopeltastes delta* (Forster) (Coleoptera: Cetoniidae); the 6-spotted bromeliad fly, *Copestylum sexmaculatum* (Palisot de Beauvois) (Diptera: Syrphidae); and several *Bombus* spp. (Hymenoptera: Apidae). There were 5 orders present in the different trap types: Hymenoptera, Diptera, Coleoptera, Lepidoptera, and Hemiptera. Because active sampling occurred by net collecting insects that were visiting the flowers, those are much more likely to be representative of the actual pollinators of *E. tampensis*. Night pollination is not a factor for this species.

The number of insects actively collected at location 1 was significantly higher than at any other location over the 3 yr period ($P = 0.0271$; F Ratio = 7.39; df = 11) (Fig. 7). However, there was no significant difference in the numbers of insects collected in each of the 3 orders (Fig. 8). Additionally, there were more blooming flowers at location 1 compared to any other location. Whereas locations 2 to 4 consisted of swamps containing pop ash (*Fraxinus caroliniana* Mill.) (Oleaceae),

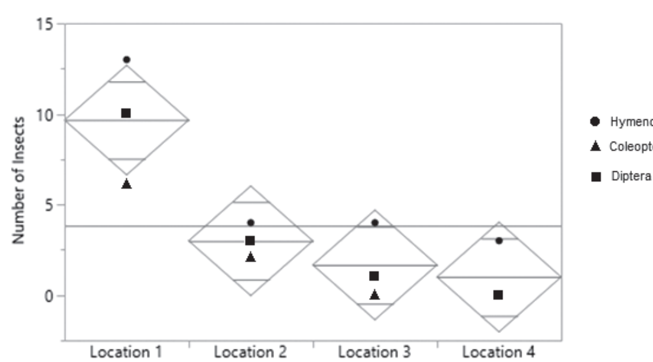

Fig. 7. Total number of insects actively sampled from each location at the Florida Panther National Wildlife Refuge over a 3-yr period (2015–2017) from *Encyclia tampensis* flowers. Location 1 had significantly more potential pollinators than did any of the other 3 locations ($P = 0.0057$), which were not significantly different from each other. Each of the points at the locations represent the insect orders Coleoptera, Hymenoptera, and Diptera, and the number of insects collected from each. Diamonds represent 95% confidence intervals for each mean.

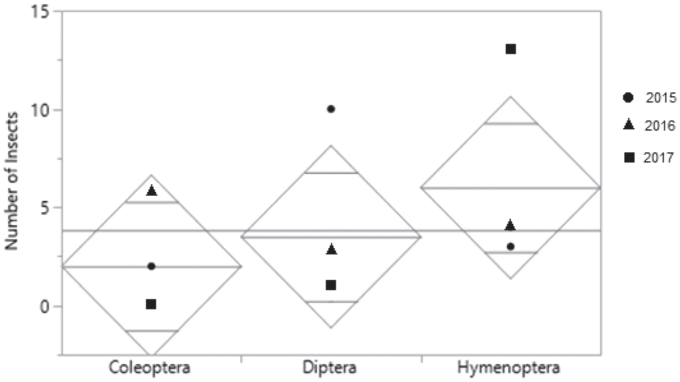

Fig. 8. Total number of insects in each order actively sampled from *Encyclia tampensis* flowers at the Florida Panther National Wildlife Refuge over a 3-yr period (2015–2017). Each of the points represents 1 of the 3 yr of sampling, and the number of insects collected in each yr. There was no significant difference between the mean total number of insects in each order that was sampled (P = 0.4142).

pond apple (*Annona glabra* Forssk.) (Annonaceae), and cypress trees (*Taxodium distichum* [L.] Rich.) (Cupressaceae), with no other blooming flowers besides the orchids, Location 1 had several different species in bloom during the sampling period (Table 2).

Insect traps were checked once daily in the afternoons, but any insects that were collected by active sampling were sorted into those collected in the morning (8:00 AM to 12:00 PM) or those from the afternoon (12:30 PM to 4:30 PM). Overall, there were 24 insect specimens captured in the morning and 22 insects captured in the afternoon over the 3-yr study (Fig. 9). At Location 1, there were almost twice as many insects collected in the morning (19) than in the afternoon (10). Three, 2, and 0 insects were collected from Locations 2, 3, and 4 in the morning, whereas 6, 3, and 3 were collected in the afternoon, respectively. Based on the results of a 2-sample t-test, there was no significant difference between the number of insects caught in the morning and the number caught in the afternoon (P = 0.439; df = 16.13; t = 0.877).

In addition to the 46 insects actively collected, there were 83 more collected from either the blue vane, WHY traps, or colored bowl traps. Of those 83, only 17 of them were species also collected during active sampling, leaving 66 specimens specific to the traps. Across all 4 locations, Hymenoptera and Diptera were the most prominent orders found in these traps (P = 0.02), with no significant difference in abundance between the 2 (P = 0.46). When comparing the orders collected in various traps, Diptera were collected only in the colored bowl traps, mostly yellow and white, whereas Hymenoptera were collected in all but blue bowls. No insects from this study were collected with attached orchid pollinia. A full list of insects identified can be found in the appendix of Ray (2018).

POLLINATOR COLLECTION – *PROSTHECHEA COCHLEATA*

Over the course of the study, only 11 insects were actively sampled from *P. cochleata* flowers. All were in the hymenopteran family Apidae. Six were *Euglossa dilemma* Friese, the non-native orchid bee found in south Florida, 3 were from the genus *Melissodes*, and 2 from the genus *Bombus*. Of these 11, none were collected from the developed location, and all were from the natural habitats. In addition to active sampling, there were 37 insects collected in the traps, only 4 of which were in the family Apidae, with the others typically being small Diptera.

Discussion

We conducted this study to begin to understand the pollination biology of *E. tampensis* and *P. cochleata* orchids in Florida. Though restoration efforts are beginning, threats of habitat loss, pests, and poaching are increasing for these orchids. Our data suggest that flowers of *E. tampensis* could be generalist pollinated by multiple insect orders, because the 3 most common genera actively collected from flowers were *Trigonopeltastes* (Coleoptera: Cetoniidae), *Copestylum* (Diptera: Syrphidae), and *Bombus* (Hymenoptera: Apidae). However, future work could be done to verify this theory if pollinators could be collected with *E. tampensis* pollinia attached to their body, because none were collected with pollinia in this study. The delta flower scarab, *T. delta*, has been recorded as a flower-visiting species across Florida and in other studies as well, including records of activity in Everglades National Park, located near our field site (Florida Panther National Wildlife Refuge) (Fontes et al. 1994; Pascarella et al. 2001). The 6-spotted bromeliad fly was recorded in south Florida from the same survey of Everglades National Park, and in a survey of Archbold Biological Station (Lake Placid, Highlands County, Florida), both categorizing it as a flower-visiting species (Pascarella et al. 2001; Deyrup & Deyrup 2012). *Bombus* spp. (bumble bees) are known generalist bee pollinators.

Whereas the same 3 genera were collected throughout the refuge, they, along with all other actively collected flower visitors, were most abundant at Location 1 at Florida Panther National Wildlife Refuge. As noted, location 1 was the developed work center and represented a disturbed habitat. Shown in Table 2, there were several species of blooming plants at location 1 that were not present in locations 2 to 4, which may have been a factor in the increased insect activity. Additionally, location 1 had a significantly higher number of flowers resulting in seed capsules during 2 of the 3 yr of the study. The higher seed capsule formation typically seen at location 1 could be the consequence of a higher number of pollinators present at that location. It is possible that planting other native, flowering plants near the edges of the swamp habitat could increase pollinator activity in those locations.

Table 2. Identified plants that were in bloom at each of the 4 locations across the Florida Panther National Wildlife Refuge that were within 10 m of blooming *Encyclia tampensis* flowers. Location 1 was a developed, landscaped work center in the refuge, whereas Locations 2 to 4 were natural swamp habitat.

	Location 1	Location 2	Location 3	Location 4
Fraxinus caroliniana (pop ash)	×	×	×	×
Annona glabra (pond apple)	—	×	×	×
Taxodium distichum (pond cypress)	—	×	×	×
Campis radicans (TRUMPET Vine)	×	—	—	—
Seville orange (bitter orange tree)	×	—	—	—
Bidens alba (spanish needle)	×	—	—	—
Allamanda cathartica (allamanda vine)	×	—	—	—
Heliconia latispatha (expanded lobsterclaw heliconia)	×	—	—	—
Catharanthus rosea (rosy periwinkle)	×	—	—	—

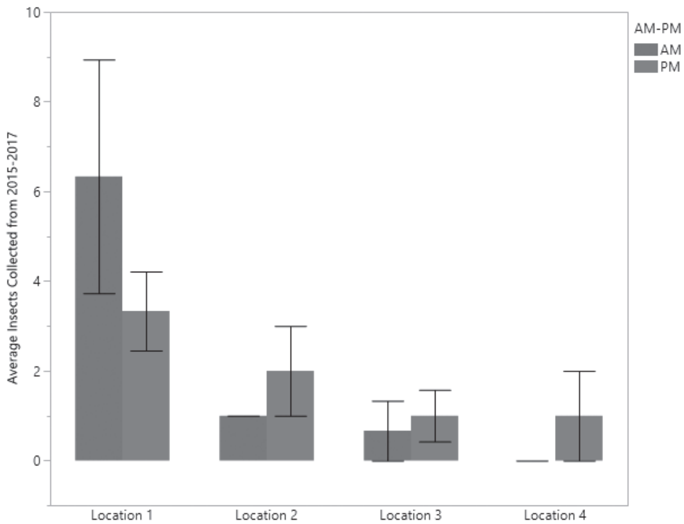

Fig. 9. Difference in average number of insects sampled around *Encyclia tampensis* flowers in the morning (8:00 AM to 12:00 PM) compared to the afternoon (12:30 PM to 4:30 PM) at 4 locations in the Florida Panther National Wildlife Refuge. A *t*-test was performed on the data and overall there was no significant difference in the number of insects collected in the morning compared to the afternoon ($t = 0.877$).

We determined that *E. tampensis* is not capable of spontaneous self-pollination. Other orchid species, such as *Epidendrum nocturnum* Jacq. (Orchidaceae), the night-fragrant orchid, are capable of either autogamy (self-pollination) or cleistogamy (self-pollination without the flower opening first) (Stort & dos Santos Pavanelli 1985; Brown 2005). Self-pollination can be advantageous for plants with a short flowering period, limited presence of pollinators, or competition for pollinators (Wyatt 1986; Snell & Aarssen 2005). A disadvantage is that self-pollination could increase the rate of inbreeding in plants, and reduce the fitness of the population (Jersáková & Johnson 2006). It is possible that there is no advantage for the evolution of self-pollination in this species, because our data suggest that *E. tampensis* is likely pollinated by a broad range of insects. After self-pollination experiments were performed on *Disa pulchra* Sond. (Orchidaceae), an orchid that is pollinated only by flies, it was found that the resulting seed capsules had about half the number of viable seeds compared to seed capsules formed from cross-pollinated plants (Jersáková & Johnson 2006). *Disa pulchra* does not provide a nectar reward for its fly pollinators, possibly causing flies to visit fewer flowers on the same plant and increasing the likelihood of self-pollination.

Ray et al. (2018) examined the floral fragrance of this orchid species and found that it was producing volatiles similar to those found in other orchid fragrance studies. This suggests that it is producing a fragrance in order to attract a pollinator. When sampling for potential *P. cochleata* pollinators, only insects in the family Apidae were actively collected. Of these, over half (6 of 11) were the non-native orchid bee in the genus *Euglossa*. However, euglossine bees typically pollinate flowers by collecting a compound used for mate attraction (Ackerman 1983). It does not seem as though the pollinia from *P*. *cochleata* would be in a suitable location to attach to a euglossine bee, suggesting that they are unlikely to be suitable pollinators of the orchid. This is because the pollinia are at the front of the flower in *P. cochleata*, whereas species that are euglossine bee pollinated have pollinia further into the flower structure.

Our study shows that whereas *E. tampensis* is not capable of spontaneous self-pollination, it is potentially pollinated by a range of flower-visiting insects. Furthermore, our data suggest that by having a variety of flowering plants nearby, pollinator activity on *E. tampensis* may be increased. Not only were more potential pollinators collected at the developed location of Florida Panther National Wildlife Refuge, but this location also produced more seed capsules from the open flowers. Further studies are underway to examine the viability of seeds from the *E. tampensis* seed capsules collected during this research. Though *E. tampensis* is not capable of spontaneous self-pollination, *P. cochleata* is readily self-pollinating at Florida Panther National Wildlife Refuge. Unlike *E. tampensis*, which had a diverse set of insect taxa collected from the flowers, insects collected from flowers of *P. cochleata* were representatives of only 1 insect family, Apidae.

Overall, this research provides a better understanding of the reproductive requirements of *E. tampensis* flowers in south Florida. Future research may be directed towards DNA analysis of gut contents of the suspected pollinators, searching for the presence of *E. tampensis* pollen. Having this information will be useful for conservation efforts for these orchids, both for protecting current populations and establishing new populations in south Florida.

Acknowledgments

We would like to thank the US Fish and Wildlife Service for allowing this research to be conducted at the Florida Panther National Wildlife Refuge in Collier County, Florida, as well as use of the facilities there. Additional thanks to James Colee at the University of Florida, Institute of Food and Agricultural Sciences, Statistics Department for statistical guidance, and to Larry Richardson and Lawrence Reeves for photography. We would like to acknowledge Andrew Stice from Illinois College, Jacksonville, Illinois, USA, for field assistance at the refuge. Finally, we thank the University of Florida Graduate School Fellowship that made this research possible.

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