

CYCAD MUTUALIST OFFERS MORE THAN POLLEN TRANSPORT¹

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Specialist insects share obligate mutualisms with some contemporary cycad species whereby the insect's pollination services are rewarded with a nursery in which the insect's larvae consume the postdispersal male cone. I prevented visits of the pollinator moth *Anatrachyntis* sp. to male *Cycas micronesica* (Cycadaceae) cones to show that consumption of the cone tissue by the mutualist hastened initiation of the plant's subsequent reproductive event. This is the first documented case where removal of a postdispersal cycad pollination organ speeds up subsequent reproductive events, and the current paradigm that the offering of cone tissue as a nursery is a sacrifice by the plant in return for the pollination services is therefore inaccurate. In *C. micronesica*, the herbivory stage of pollination mutualism confers a cryptic benefit of cone tissue disposal, which translates into an increase in ultimate lifetime reproductive effort. The plant population relies on the pollinator for moving gametes, as well as for increasing the number of male coning events. The dual benefits afforded to the plant by associating with this pollinator shows that mutualism can operate simultaneously on very different traits.

Keywords: *Anatrachyntis*; Cycadaceae; *Cycas micronesica*; mutualism; plant–insect interaction; pollination.

Cycads are dioecious gymnosperms, among the most ancient of extant spermatophytes, exhibit many primitive features, and the study of their pollination systems may shed light on the origins of plant and animal interdependence (Norstog and Nicholls, 1997). Primary growth of most cycads occurs in recurrent flushes with pulses of vegetative or reproductive structures emerging from stem apices to rapidly attain mature size. During the reproductive flush of many cycad species, insect pollinators enter loosened sporophylls of mature male cones (Fig. 1A; Tang, 2004). The sporophylls produce heat and chemicals that may modify the behavior of the pollinators (Seymour et al., 2004; Tang, 2004; Terry et al., 2007). Pellmyr et al. (1991) contend that cycad cone volatiles that serve as chemical attractants for pollinators may have evolved from herbivore deterrents. Male cones house the pollinators for a stopover, and in at least one species of cycad, *Macrozamia lucida*, the pollinators are then forced to evacuate by an ephemeral increase in volatile emissions to potentially lethal dosages (Terry et al., 2007). In the *Cycas* pollination syndrome, female pseudocones use mimicking olfactory cues to attract pollinators.

The Mariana Island Archipelago is home to the cycad *Cycas micronesica* K. D. Hill (Norstog and Nicholls, 1997). The microlepidopteran *Anatrachyntis* sp. (Cosmopterygidae) (Fig. 1B–D), several closely related species of *Carpophilus* sp. beetle (Nitidulidae), and one species of staphylinid beetle (Marler and Muniappan, 2006; Terry et al., in press) are the most common insects that visit both male cones and female pseudocones. Pollen-bearing *Anatrachyntis* moths increase seed set when offered to receptive pseudocones (L.I. Terry [University of Guam] and T. E. Marler, unpublished data). The diel thermogenesis cycles of cones of this taxa are unlike previously published diel cycles,

with metabolically generated increases in cone temperature occurring throughout the day and a late afternoon peak temperature increase of 4.5°C above ambient (Roemer et al., in press). *Cycas micronesica* cone volatiles are characterized as fruity, and the major components are methyl isovalerate (ester) and linalool (monoterpene) (Terry et al., in press).

This pollination mutualism of cycads regulated by the cone's thermogenesis and volatiles provides effective pollination for the plant population, but a second phase unfolds as the cone's pollen supply wanes. The male cones become brooding facilities for the insects (Norstog and Fawcett, 1989; Tang 2004). Indeed, ovipositions within male cones, but not female cones, during the pollination phase give rise to a postdispersal pollinator nursery, and the integrity of the cone is rapidly compromised as tissue is devoured and portions or all of it collapse. The *Anatrachyntis* moth is the only known male cone consumer of *C. micronesica* (Marler and Muniappan, 2006; Terry et al., 2009). This nursery phase has been documented for the majority of cycad species for which pollination biology has been studied, but its adaptive significance during evolution of this mutualism and its role in contemporary cycad biology have been considered the plant's sacrifice of the organ in support of the animal (e.g., Seymour et al., 2004).

I tested whether plants with cone tissue removed by *Anatrachyntis* larvae would exhibit subsequent growth patterns that differed in timing from those of plants in which cones were protected from *Anatrachyntis* herbivory. I also examined the influence of plant sex and relative size of the local plant population within a habitat on frequency of reproductive events.

MATERIALS AND METHODS

Male cone manipulations—I used two methods to prevent pollinator visits to male *Cycas micronesica* cones (Fig. 1A) in March 2002. The chemical method consisted of twice weekly sprays of chlorpyrifos for 1 wk before and 1 wk after pollen release. The physical method involved a screen barrier that restricted pollinator access. A third manipulative treatment consisted of the chemical spray, followed by manual removal of the cone at the same time as the loss of integrity of control cones. This treatment enabled me to determine

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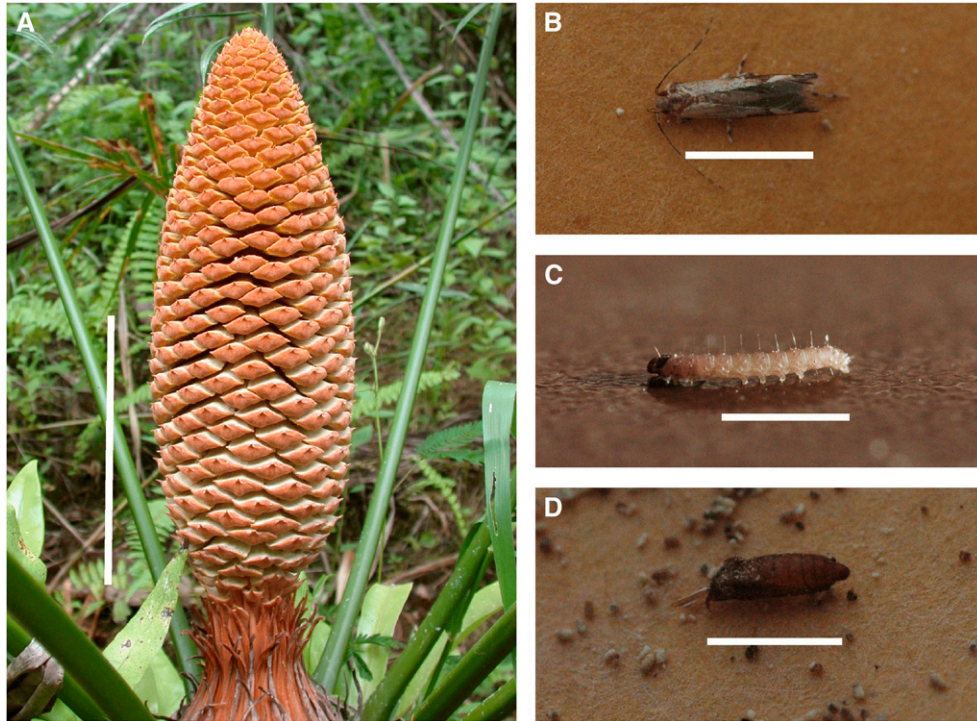


Fig. 1. (A) Male *Cycas micronesica* cone at pollen dispersal stage showing loosened sporophylls between which *Anatrachyntis* adults enter; scale bar = 20 cm. *Anatrachyntis* (B) adult dorsal view, (C) larvae, and (D) pupae; scale bar = 4 mm.

if timing of cone removal was the means by which pollinator behavior influenced subsequent plant behavior. Control plants were allowed access to natural pollinator visits and the consequent cone consumption. These four levels of the fixed qualitative treatment variable were replicated 15 times. Plants in each treatment were selected in a random design to prevent sampling bias. I visited each plant monthly and recorded the dates of vegetative and reproductive flushing events. I followed each plant through two subsequent coning events.

Effect of plant sex and habitat on coning frequency—I marked male and female plants as they initiated reproductive events from January through June 2002 in four habitats in different parts of Guam. These included three locations within contiguous northern coastal limestone forests and one location in a fragmented southern ravine forest. My goal of 15 individuals for each sex and habitat was not achievable for every habitat within the 6-mo period I defined as the beginning of the study. The experiment therefore had unequal replications: northwestern, 14 males, 15 females; northern, 14 males, 15 females; northeastern, 13 males, 15 females; southern, 10 males, 11 females. I visited each plant monthly until the subsequent reproductive event.

Statistical analyses—Male cone manipulations—The three response variables were (1) number of weeks to leaf emergence, (2) number of weeks to cone emergence, and (3) number of leaf flushes between successive coning events. For (1) and (2), the experimental design was a split-plot completely randomized (CR) design where the split-plot factor was treated as repeated measures. The treatment design was a fixed qualitative (four cone manipulation levels) \times qualitative (two cycles) factorial. Software was the PROC MIXED procedure in the program SAS 9.13 (SAS Institute, Cary, North Carolina, USA). All ANOVA assumptions were met without violations. Pairwise mean comparisons were based on Tukey's honestly significant difference for the eight treatment combinations.

For the number of leaf flushes between coning events, only two values (1 or 2) were recorded. The variable was therefore treated as binary. Advanced analysis of the binary repeated variable was a split-plot, CR design where the split-plot factor was treated as repeated measures. The treatment design was a fixed qualitative (four cone manipulation levels) \times qualitative (two cycles) factorial. Software was the PROC GLIMMIX procedure in SAS 9.13.

Plant sex and habitat coning frequency—Experimental design was CR with unequal replications, and treatment design was a fixed qualitative (four habitat levels) \times qualitative (two sex category levels) in a factorial. Software was the PROC GLM and PROC MIXED procedures in SAS 9.13. When violations of ANOVA assumptions were examined, unequal variance of error distribution was observed between male and female plants. To correct this unequal error variance, a heteroscedasticity-adjusted ANOVA was performed with the SAS MIXED procedure, which resulted in a random distribution plot in the residual to confirm the correction of the unequal variance. Pairwise mean comparison was based on Tukey's method for the levels within each main effect.

RESULTS

Analysis of the 4×2 split-plot repeated measures ANOVA with AR(1) covariance structure indicated that the two main effects were significant, as was their interaction for weeks to leaf emergence ($F_{3, 56} = 18.80, P < 0.0001$) and for weeks to cone emergence ($F_{3, 56} = 13.75, P < 0.0001$). Plants in which the pollinators were denied access to pollen-dispersing cones by means of chemical sprays or screen enclosures initiated a leaf flush at about 26 wk and a subsequent coning event at 57–60 wk (Fig. 2). In contrast, control plants experiencing natural consumption of cone tissue by pollinator larvae and plants from which chemically protected cones were removed manually initiated a leaf flush at 14 wk and a subsequent coning event at 39 wk. The time between the first and second reproductive cycle did not differ among the treatments. For the number of leaf flushes between sequential coning events, the main effects and their interaction were not significant.

Natural phenology of male and female plants in several habitats throughout Guam indicated the interaction of habitat with sex was not significant. However, the habitat main effect

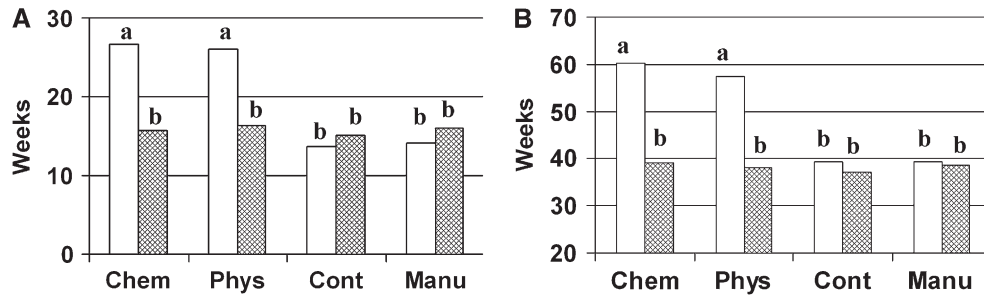


Fig. 2. The number of weeks until initiation of growth events for *Cycas micronesica* plants on Guam. Cycles of emergence following chemical (Chem) or physical (Phys) protection from pollinators, control (Cont), and manual removal of cone following chemical protection (Manu). (A) First (open bars) and second (cross hatch bars) cycles of leaf emergence. (B) Cycles of cone emergence on same plants. Bars with different letters are significantly different, based on Tukey's honestly significant difference ($\alpha = 0.05$).

($F_{3,66} = 8.32, P < 0.0001$) and sex main effect ($F_{1,74} = 54.43, P < 0.0001$) were both highly significant. Sequential coning in plants from the small forest fragment in southern Guam was 62.2 wk (Fig. 3A: S), but that from coastal forests throughout northern Guam was 50.3 wk (Fig. 3A: NW, N, NE). The average window of time that separated sequential male cones was 70% of that for sequential female pseudocones (Fig. 3B).

DISCUSSION

Removal of the male cone by pollinator herbivory altered the time for these male plants to disperse pollen again. Cone removal by the mutualist shortened the time required to potentially sire seeds in the next reproductive cycle to 66% of that for the plants that retained intact cones after pollen dispersal. Integration of this shortened intertermating interval over the life of these long-lived plants greatly increases the cumulative number of a male plant's potentially sired seeds as a direct result of cone consumption by the pollinator.

The contemporary belief that cone herbivory is antagonistic and is the cost to the plant in return for pollination services is inaccurate and may have arisen because it seems credible. Research into pollination of cycads has been extensive since the 1980s when studies validated that specialist insects are pollinators of *Zamia* species (e.g., Norstog and Fawcett, 1989; Tang, 1987). These relatively recent findings reversed the long-standing erroneous belief that all cycads are wind-pollinated (see Norstog and Nicholls, 1997; Tang, 2004). In response, the research community may have been overly focused on uncovering the intricacies of the pollination stage of this mutualism. As

a result, these previous studies have not taken a long enough perspective to fully understand the mutualism.

Community ecology is defined by spatiotemporal fluctuations of resources. For the *Cycas* pollination mutualist, the obligate resource for success of subsequent generations is the mature male cone. Smaller forest fragments may have fewer male cones due to the limited local plant population, and the number of plants with cones is highly seasonal. The pollination syndrome may therefore be driven by a dual regulation: recent history of local cycad male cone availability regulates the size of the pollinator population, and local pollinator population coincident with a plant's pollen dispersal stage regulates the speed of subsequent primary growth for each male plant.

The influence of habitat on coning frequency (Fig. 3A) further informs this discussion. Three of the four sites were located in the northwestern, northern, and northeastern habitats that were within the contiguous cycad habitat that extends around the coastline of the northern half of the island. From Guam's northern tip, this native forest habitat extends 15 km down the west side of the island and 36 km down the east side. The fourth habitat was located in a southern forested ravine that is typical of the riparian forest fragments in the southern part of the island that are isolated by extensive savannah habitat. This particular fragment was ca. 1900 m long and 160 m wide at the widest point. The average return time for sequential coning in plants from the isolated forest fragment with relatively fewer available cones in southern Guam was 62.2 wk (Fig. 3A: S), but was 50.3 wk from contiguous coastal forests with copious cone displays throughout northern Guam (Fig. 3A: NW, N, NE). Moths working the high-density plant community throughout the three northern habitats that are connected with contiguous forest

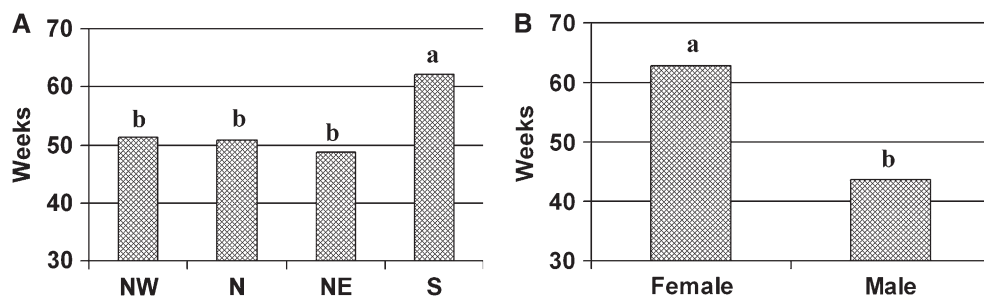


Fig. 3. The number of weeks between sequential coning events for *Cycas micronesica* plants on Guam. (A) The influence of habitat (northwestern, northern, northeastern, or southern Guam), means of male and female plants. (B) The influence of gender, means of four habitats. Bars with different letters are significantly different, based on Tukey's honestly significant difference ($\alpha = 0.05$).

would have access to many more nursery facilities for recruitment purposes than would moths working the fragmented forests characterized by the southern habitat.

My results demonstrate that the cone is communicating with the stem apex in a manner that controls stem behavior. I suggest two possible nonexclusive mechanisms. First, the male cone may use a positive signal that suppresses primary growth. Partial or complete cone removal partially or completely removes the suppressive signal. A parallel is the regulation of angiosperm fruit retention and consequent subtending stem behavior by the organ's hormone synthesis and basipetal export. The monopodial tendency of *Cycas micronesica* plants (Niklas and Marler, 2008) indicates a strong apical dominance is characteristic of the taxa, possibly mediated by auxin basipetal transport. Unfortunately, no empirical data are available to illuminate this discussion for any cycad species. Second, the communication may be one of the resource trade-off phenomena among life history traits. Construction of the *Cycas* male cone is expensive not only because of the biomass, but also because costly lipids and starch are stored for use as substrates for thermogenesis (Tang et al., 1987). The delay in the onset of future reproductive growth after a cone is denied access to its pollinators may signify a cost resulting from sustained maintenance of the cone tissue after pollen dispersal within the context of an optimal-allocation model (Iwasa and Cohen, 1989). Empirical and anecdotal evidence lends support to this resource trade-off scenario through demographic intersexual dimorphism. On Guam for example, the average window of time that separates sequential male cones is 70% of that for sequential female pseudocones (Fig. 3B). Guam's female plants allocate resources to developing seeds for 30+ mo (Marler et al., 2006), which is in sharp contrast to the 8–9 wk at which a male plant terminates allocation of resources to cone development. Long delays in subsequent growth after emergence of female cones, but not male cones, have been reported for numerous cycad species (Norstog and Nicholls, 1997).

The independent influences of herbivory and pollination on potential plant fitness have been extensively studied, but little has been reported on the integration of herbivore- and pollinator-mediated physiological, developmental, and evolutionary responses. The few attempts to integrate simultaneous pollinator and herbivore interactions with plants portray herbivory and pollination as opposing interactive forces (Herrera et al., 2002; Strauss and Armbruster, 1997). For example, the yucca (Crabb and Pellmyr, 2006), fig (Weiblen, 2002), and cactus (Holland and Fleming, 1999) mutualisms with obligate pollinators characterized by the exchange of pollination services for larvae food have been portrayed as examples of evolutionary conflict in which the herbivory is a necessary cost of pollination. I have shown that in some cases herbivory and pollination work in tandem on distinct traits to improve potential plant fitness. Pollination bestows direct benefit to current seed set, then disposal of the reproductive structure confers an ability to increase frequency of sequential coning events. Access to this service of rapid tissue disposal by the arthropod may be the reason male *Cycas* cones did not evolve organ abscission capabilities.

This is the first documented case where removal of a postdispersal cycad pollination organ speeds up subsequent reproductive events, and similar cases may be found in flowering plants. Further research is needed to determine whether this response is idiosyncratic or a general phenomenon. Rather than studies to accumulate eclectic empirical evidence with other case studies, more benefit may be obtained by addressing various models

and hypotheses. For example, questions about the evolution of life histories (Roff, 1992) may address how the influence of variation in life history traits correlates with the impact of post-dispersive pollination organ removal. Evaluating fitness using methods of antagonistic pleiotropy (Arnqvist and Rowe, 1995) and dynamic state variable models (Clark and Mangel, 2000) may uncover generalized constraints and trade-offs involved in removal of reproductive structures after their direct reproductive role has been completed. The costs-of-reproduction hypothesis (Obeso, 2002) in particular should be revisited by studies that consider multiple seasons. Static estimates of reproductive costs are now known to be inaccurate, yet even studies that included dynamic estimates to quantify the cost of floral nectar, carbon contributions by photosynthesis of reproductive structures, or compensatory increase in photosynthesis of nearby leaves (Southwick, 1984; Ashman, 1994; Obeso, 2002) have ignored the possible influence of costs of maintaining postdispersive structures on future reproductive behavior. Further, studies exposing the need to quantify resource reabsorption from current reproductive structures after dispersal of pollen (Ashman, 1994) have also failed to consider influences on subsequent reproductive effort.

The misperceptions about *Cycas* pollination mutualisms should be addressed because the herbivory stage of the mutualism is not a plant sacrifice offered within an antagonism, but is a cryptic benefit of cone tissue disposal realized as an increase in ultimate lifetime reproductive effort. The plant population relies on the pollinator for gamete receipt and export, but also for increasing the number of male coning events. The distinct benefits of herbivory and pollination show that mutualism can operate simultaneously on very different traits. Mycorrhizae and nitrogen-fixing symbionts also influence plant fitness (Barea et al., 2002; Parker, 1995), and the particulars of cycad mycobiont and cyanobiont biology have received less attention than those of pollination mutualist biology. Until we grasp the nuances of these mutualisms more fully, we will not fully appreciate how mutualisms evolved in this ancient spermatophyte group. More important, these deficiencies in our understanding of obligate mutualisms limit our ability to assign causal mechanisms to contemporary threats or to define conservation management protocols for the >50% of cycad species that are threatened (Donaldson, 2003), especially those taxa for which in situ conservation efforts are still feasible.

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