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Diversity in *Cycas* (Cycadales: Cycadaceae) Species Offered as Larval Food Influences Fecundity of *Chilades pandava* (Lepidoptera: Lycaenidae) Adults

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ABSTRACT: *Chilades pandava* (Lepidoptera: Lycaenidae) larval food quality was studied to determine its influence on adult life history traits. A wild population from *Cycas nongnoochiae* (Cycadales: Cycadaceae) endemic habitat behaved similarly to the population collected from a garden setting. *Cycas micronesica, Cycas revoluta*, and *Cycas seemannii* leaves were used as high-quality food, whereas *C nongnoochiae, Cycas taitungensis*, and *Cycas condaoensis* leaves were used as low-quality food. The daily oviposition rate was not influenced by food quality, but longevity and lifetime fecundity of females were increased by high-quality larval food. These results indicate that in situ *Cycas* species impose a physiological constraint on the genetic capacity to produce offspring by *C pandava*. The removal of that constraint by high-quality novel *Cycas* species may be one reason this butterfly can increase in population rapidly after an invasion event and express greater herbivory of *Cycas* species within invaded regions.

KEYWORDS: Dietary constraints, fitness, Lepidoptera, reproductive success

Introduction

The study of life history traits such as life span and fecundity is of fundamental importance for applied ecology and understanding how natural systems function. Moreover, when a plant species becomes endangered due to damage by an invasive phytophagous insect, more knowledge about life history traits of all trophic levels may inform management decisions.

Chilades pandava (Lepidoptera: Lycaenidae) is a butterfly that has a wide indigenous range and is characterized by several discrete subpopulations delineated by geography.[1] Larval food for this Lycaenid species is young, expanding leaf tissue of *Cycas* L. (Cycadales: Cycadaceae) species. The extent of herbivory is highly disparate among various *Cycas* species when the plants are in a common garden (Figure 1), with the species that share native habitat with a Lycaenid species exhibiting less damage than the naïve species.[2] The plant traits that mediate these dynamics are not understood. Availability of quality food during early development of animals may exert a strong influence on longevity, organ function, or fecundity during adult life stages.[3] Herbivorous insects in particular reveal a strong fecundity response to food quality.[4] But nothing is known about differences in *C pandava* larval food quality among the many *Cycas* species and how that influences life history traits of the butterfly.

Increased knowledge about *C pandava* biology and ecology regarding food quality may have 3 applications. First, within the native range of the butterfly,[1] greater understanding of how the phytophagous insect and its native host *Cycas* species coexist in natural habitats is needed. An example of this is *Cycas nongnoochiae* K.D. Hill, within its restricted endemic range.[5] Moreover, different geographic subpopulations of a phytophagous arthropod may exhibit different host plant specializations, indicating that each species pair may exhibit unique interactions. Indeed, conservation of cycad species is important partly because they embody a critical function in the life histories of other organisms.[6–8] Second, within insular regions where this butterfly has invaded,[1] increased knowledge about how the phytophagous insect threatens a native *Cycas* species will improve conservation decisions for the plant. An example of this is *Cycas micronesica* K.D. Hill on the island of Guam.[9] Third, in botanical garden settings with multiple *Cycas* species represented in the germplasm, the mixed diet of this butterfly may be controlled by variation in chemical, phenological, and structural traits among the many *Cycas* species. An example of this is Nong Nooch Tropical Botanical Garden where 112 of the world's *Cycas* species are grown in a common garden setting.[2]

In this study, we addressed the hypothesis that *Cycas* species diversity as *C pandava* larval food should affect life history traits of the adult butterfly. Our primary objective was to determine the influence of *Cycas* food quality (heavily damaged versus minimally damaged species) during the *C pandava* larval stage on key life history traits of the adult stage. Our secondary objective was to determine whether the behavior of the *C pandava* subpopulation that evolved exclusively on *C nongnoochiae*
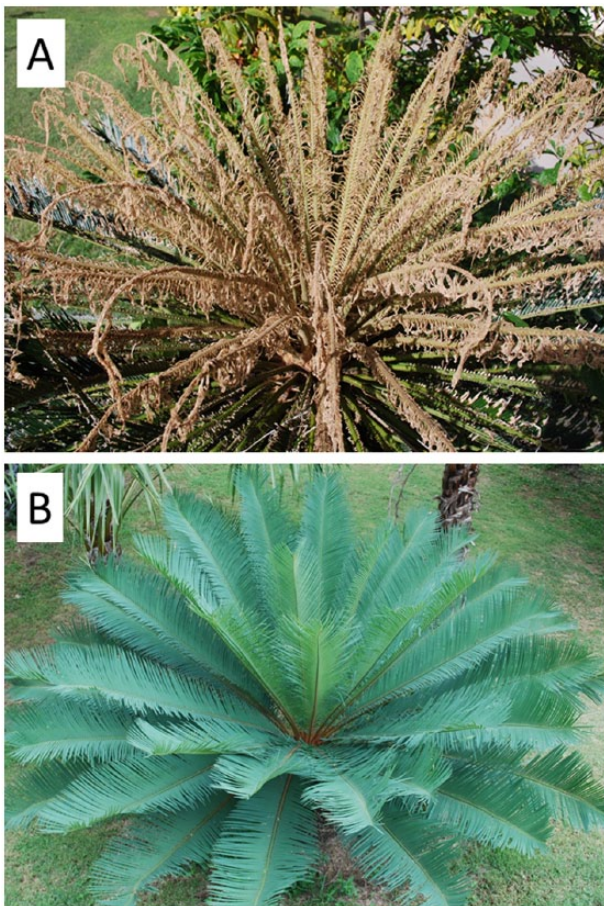
Figure 1. Herbivory of *Cycas* leaves in the presence of *Chilades pandava* can be acute or minimal, as determined by the plant species. (A) *Cycas revoluta* is the most widely cultivated *Cycas* species worldwide and exhibits extreme damage by the butterfly larvae. (B) Minimal herbivory of *Cycas pachypoda* leaves occurs despite heavy butterfly pressure in a common garden setting.

as its host would differ from that of a mongrel population with a long history of experience with numerous *Cycas* species within a botanic garden setting.

Materials and Methods

Collection of larvae

Chilades pandava larvae were collected in Tak Fa, Thailand, June 18 to 19, 2013, within the endemic range of *C nongnoochiae*. They were transported to Nong Nooch Tropical Botanical Garden in 15.6-L plastic containers while being fed with *C nongnoochiae* leaves collected in habitat. The larvae were then reared in the same plastic containers and fed ad libitum with expanding *C nongnoochiae* leaves collected from the Garden's plants as the only food resource until pupation. This population is referred to as the "wild" population hereinafter. *Chilades pandava* larvae were collected from various *Cycas* plants within the Garden on June 21, 2013. They were placed in 15.6-L plastic containers and fed ad libitum with a mixture of expanding *C micronesica*, *Cycas seemannii* A. Braun, and

Cycas edentata de Laub. leaves until pupation. This population is referred to as the "garden" population.

The experimental procedures that employed caged butterfly adults were conducted on the top of plant production benches within one of the nursery sections of the Garden. The open-sided facility was covered with a polycarbonate corrugated roof that transmitted sunlight but excluded rain. A shaded maximum/minimum thermometer was installed and data were recorded daily. The mean daily minimum was 26.7°C, and the mean daily maximum was 32.8°C for the duration of the experiment. Mean time of sunrise was 05:59 AM, mean time of sunset was 06:43 PM, and mean photoperiod was 12.72 hours. The plastic containers with developing larvae were inspected daily for pupae. Following pupation, each pupa was transferred to 0.072-m³ cages made of white polyester fabric with 130 holes per centimeter. This first generation of caged butterflies was allowed to comingle and mate freely within one cage for each of the populations. They were fed with commercial honey by placing pieces of sponge soaked in the honey on top of the cages.

Production of experimental units

The collection of eggs to begin the second generation of caged butterflies was initiated immediately by placing leaf sections from expanding leaves into the cages for 1 to 2 hours per day. The leaf sections included 15 to 20 cm of rachis and expanding leaflets. The basal leaflets were removed to allow the rachis to be placed into 180-mL plastic cups with enough water to keep the base of the rachis section wet. The apical leaflets were selected by the female butterflies for ovipositioning. The *Cycas* species were selected from the list detailed below.

We employed 2 larval food quality levels. High-quality food was defined as leaf sections from *Cycas* species that exhibited extreme *C pandava* herbivory levels within the Garden.[2] Low-quality food was defined as leaf sections from *Cycas* species that exhibited minimal or no observable herbivory within the Garden. For both populations of butterflies, the high-quality food comprised a mixture of *C micronesica*, *Cycas revoluta* Thunb., and *C seemannii* leaves. These were novel food substrates for the wild population of butterflies. For the wild population, we used *C nongnoochiae* leaves as the exclusive taxon for low-quality food. For the garden population, we used a mixture of *C nongnoochiae*, *Cycas taitungensis* C.F. Shen, K.D. Hill, C.H. Tsou, and C.J. Chen and *Cycas condaoensis* K.D. Hill and S.L. Yang as the low-quality food.

High-quality and low-quality leaf sections were offered within each cage daily. Every leaf section contained numerous eggs when removed from the first-generation butterfly cages, and they were placed into separate cages for hatching. The water in the cups was changed at least daily until eggs hatched. When larvae were present, the leaf section was removed from the water and placed on a bed of fresh leaf material in 15.6-L

plastic containers. This allowed the larvae to forage freely among the food resources that were made available. New 15.6-L containers were prepared with a bed of fresh leaf material every morning and then partially consumed leaf sections containing the developing larvae were transferred from the previous day's container to the fresh food source. All larvae were fed ad libitum until pupation.

The containers were inspected daily for pupae, and when present, the pupae were removed from the plastic containers and placed into 0.072-m³ cages. Our goal was to create 10 experimental units from each of 2 populations and each of 2 larval food quality levels. Therefore, we had multiple cages for each of the 4 treatment combinations, with each containing multiple pupae. The cages were inspected daily, and when adults emerged, their adult age was defined as day 0. Newly emerged butterflies were distributed to 0.072-m³ cages such that each cage served as an experimental unit with 1 female and 2 males of the same age.

Our preliminary work in 2012 revealed that wild-collected adults of this species do not adjust to the stress imposed by the cages, as they remain idle until death. But adults that emerge from pupa within the cages will feed, mate, and oviposit freely. Therefore, our methods standardized all of the experimental units from both butterfly populations. The parents of every experimental unit were first-generation caged butterflies, and the actual butterflies used to measure life history traits were second-generation caged butterflies. The length of time from egg to pupation was 7 to 11 days for the garden population and 9 to 11 days for the wild population. Duration of pupae stage was 4 to 7 days for both populations.

Performance measurements

The butterflies were provisioned with honey using soaked sponges placed on the cage fabric as the means of delivery. Beginning the initial day, the butterflies in each cage were offered a *Cycas* leaf section to quantify egg production by the single female. The *Cycas* species were selected at random among the days, but within 1 day, all 40 experimental units were provisioned with the same species of *Cycas* for accepting the eggs. Following 1 to 2 hours of access, the leaf sections were retrieved and eggs were counted with a handheld magnifying lens. Health of each butterfly was observed, and date of death was recorded for each of the original 3 animals. The extent of polyandry in this species is not known, so when death of a male occurred, we immediately replaced the experimental unit with another male such that each female was always provided access to 2 males. Replacement males were marked with a permanent marker on one of the wings to distinguish them from the original 2 males. The life span of the replacement males was not recorded. When the female butterfly died, no more measurements were obtained from the experimental unit.

The reproductive performance of each female was quantified with the pre-oviposition period, the reproductive period, the daily oviposition rate, the post-oviposition period, the total lifetime egg production, and the total life span. Total life span was the only trait recorded for the males. The life span of the 2 males within each experimental unit was averaged to provide one number.

Statistical analyses

Prerequisites for parametric tests were met with the exception of nonhomogeneous variances for female life span and reproductive period (according to Levene test and the Brown-Forsythe test). Therefore, a mixed linear model was used (SAS Version 9.3, PROC MIXED) with nonequal variances set for female life span and reproductive period and homogeneous variances set for the remainder of the response variables. A 2-factor factorial with populations (wild and garden) and food quality (high and low) designated as the independent sources of variation.

Results

The population × food quality interaction was not significant for pre-oviposition period, oviposition period, post-oviposition period, total life span, oviposition rate, or lifetime fecundity of females, or life span of males. Therefore, comparing differences in the behaviors of the 2 populations was unnecessary. The influence of food quality on the fecund and nonfecund time periods of the females was dissimilar. Pre-oviposition period did not differ between the populations (P = .6231) or the food qualities (Table 1), and the mean was 1.8 days with a range of 0 to 6 days. Similarly, the oviposition period did not differ between the populations (P = .8971) or the food qualities (Table 1), and the mean was 22.2 days with a range of 3 to 41 days. The post-oviposition period also did not differ between the populations (P = .7132), but females fed with high-quality food lived 3.8 times longer after they became nongravid than females fed with low-quality food (Table 1). The post-oviposition period was longer than the pre-oviposition period for the females fed with high-quality food, but the reverse was true for the females fed with low-quality food. Total life span of females was not influenced by population (P = .9036), but females fed with high-quality food lived 6.5 days longer than females fed with low-quality food (Table 1). Total life span of males was not influenced by population (P = .1402) or food quality (Table 1). Males exhibited much shorter life span than females, exhibiting a mean of 14.9 days and a range of 5 to 32 days.

The oviposition rate was not influenced by population (P = .6707) or food quality (Table 1). These small butterflies produced a mean of 24 eggs per day. To observe the relationship between oviposition period and the fecundity rate, we generated a scatterplot (Figure 2A) that revealed a unimodal pattern with oviposition periods close to the median associating with higher

Table 1. Life history traits of *Chilades pandava* adults fed with high-quality substrates (*Cycas micronesica, Cycas revoluta, Cycas seemannii*) or low-quality substrates (*Cycas nongnoochiae, Cycas taitungensis, Cycas condaoensis*) during the larval stage.

TRAITS EVALUATED (MEAN±SE)	HIGH QUALITY	LOW QUALITY	P VALUES OF THE ANOVAS
Pre-oviposition period (day)	1.9±0.3	1.8±0.3	.8057
Oviposition period (day)	24.5±2.2	19.9±2.1	.1429
Post-oviposition period (day)	2.3±0.5	0.6±0.5	.0136
Oviposition rate (eggs/d)	27.1±2.7	20.8±2.6	.1039
Female life span (day)	28.7±2.0	22.2±2.1	.0308
Male life span (day)	15.9±1.1	13.9±1.1	.2067

Abbreviation: ANOVAs, analyses of variance.
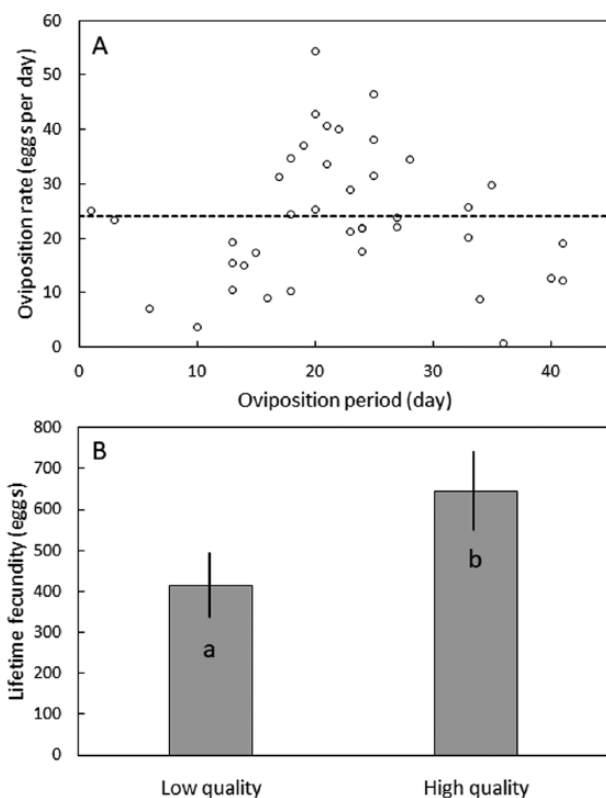
Figure 2. (A) Scatterplot of oviposition rate versus oviposition period for *Chilades pandava* females. Dashed line is overall mean of 24 eggs per day. (B) Lifetime fecundity of *C pandava* females after being fed with high-quality substrates (*Cycas micronesica, Cycas revoluta, Cycas seemannii*) or low-quality substrates (*Cycas nongnoochiae, Cycas taitungensis, Cycas condaoensis*) during the larval stage. Mean±SE, n=20. Bars with different letters are significantly different at 1% level according to analysis of variance.

fecundity rates. Therefore, a life span that deviates substantially from average may be a distinct second physiological constraint that reduces fitness below genetic capacity. The multiplicative effect of the nonsignificant 23% increase in oviposition period and nonsignificant 30% increase in fecundity rate that resulted from high-quality food led to a 55% increase (P = .0241) in lifetime fecundity (Figure 2B). Lifetime production exceeding 500

eggs was exhibited by 58% of the females, and lifetime production exceeding 1000 eggs was exhibited by 8% of the females.

Discussion

We have shown for the first time that *Cycas* species which are known to exhibit extreme damage in the presence of *C pandava* populations improve fecundity of butterfly adults when their leaves are employed as larval food. The female adults lived longer and produced more eggs if the larvae were fed with leaves of these *Cycas* species, none of which coevolved with a phytophagous Lycaenid. The predictions from our primary objective were confirmed, and larval food quality does influence life history traits of the adults, primarily by increasing their performance in producing offspring.

We have also shown that this response was not influenced by decades of access to many novel *Cycas* species by a butterfly population. Adult life history traits of the mongrel butterfly population within Nong Nooch Tropical Botanical Garden and the wild butterfly population associating with the endemic *C nongnoochiae* responded similarly to *Cycas* larval food choices. We added this secondary objective because *C pandava* female adult oviposition choices were beneficial for offspring in the wild population but were muted in the experienced Garden population which was fed with a mixed diet.[10] The predictions from our secondary objective were not confirmed, and the benefits from high-quality larval food to adult fecundity were realized in a similar fashion for both populations of butterflies.

Ecology of endemic habitats

These results have relevance toward greater understanding of how an in situ population of *Cycas* can coexist with a specialist phytophagous Lycaenid. A singular generation of larvae reared on *C micronesica*, *C revoluta*, and *C seemannii* leaves produced *C pandava* females that exhibited a 1.55-fold increase in egg production above that of larvae reared on *C nongnoochiae*, *C taitungensis*, and *C condaoensis* leaves. These data illuminate for the first time that the genetic capacity of an adult female to produce offspring is physiologically constrained by feeding on its

defended in situ host plant, and feeding on an alien host plant removed this physiological constraint. The ability of the native host plant to impede the capacity of females to produce eggs via food quality traits may be one means of keeping the in situ specialist phytophagous insect population constrained.

Conservation within invaded habitats

These results also increase our knowledge of how an invasive phytophagous Lycaenid can cause greater damage to a naïve *Cycas* population than might be predicted from observing the phytophagous insect within its native range. Very recent invasions of this *Cycas* pest in Micronesia[9] and Africa,[11] for example, provide the butterflies with a diet that lacks the physiological constraints imposed on the butterfly within its native range. These issues likely contribute to the exponential growth curve of the butterfly population immediately after an invasion and the greater herbivory damage observed within the invaded locations.

Garden settings

These results also add to our ability to understand the butterfly behavior within an ex situ botanic garden setting where numerous *Cycas* species are being grown. That a specialist phytophagous insect would exhibit compromised fitness when reared on its coevolved host plant but increased fitness when reared on a novel substrate seems counter-intuitive. For in situ butterflies with a single host *Cycas* species (eg, Thailand's *C nongnoochiae* habitat), the mothers are not exposed to complicated choices for oviposition sites. But in botanic gardens, the butterfly mothers are confronted by numerous *Cycas* species with a wide range of larval food quality. Egg-laying decisions in insects can dictate the environmental quality that defines the well-being of their offspring and is a form of parental care.[12] The complicated means by which this phenomenon transpire may be the reason that active oviposition decisions by wild mothers confirmed a preference for the high-quality *Cycas* species, but this behavior became muted over time as generations of butterflies became accustomed to access to numerous *Cycas* species.[13]

Caveats

Studies to characterize life history traits of phytophagous specialist insects should exercise caution while interpreting results. Our study has shown that life history responses to an in situ food source can surprisingly underestimate performance capacity of the insect. If a phytophagous insect is provided a novel food choice, their life history traits may improve.

The differences in *C pandava* herbivory that were reported among 85 *Cycas* species[2] were based on observations of the germplasm following chronic weekly pesticide applications. These prophylactic pesticide applications likely reduced the magnitude of observed damage between susceptible versus tolerant species. The susceptible species may exhibit more damage without the pesticide regime, whereas the tolerant species would likely not show a change in damage.

Future research

Our results have illuminated other avenues for continued discovery. For example, the herbivory deterrence exhibited by *C nongnoochiae*, *C taitungensis*, and *C condaoensis* may be structural or biochemical. For a structural defensive mechanism to exert such a profound physiological response on the butterflies is highly unlikely, so we suspect that biochemical herbivory deterrence is causal. This could be experimentally determined. Moreover, the factors that affect this bitrophic relationship are complicated, as we have opined elsewhere,[13] and there is no reason to believe that one factor mediates 100% of the interplay between these 2 organisms. In addition to structural or chemical defense, we believe diversity in phenology of leaf growth among *Cycas* species may also mediate some of the responses. This also could be experimentally tested.

Our methods were restricted to the bitrophic relationship. But the dynamics between the *C pandava* population and its natural enemies may also be under the influence of the larval food quality. Therefore, tritrophic relationships need to be studied to fully understand the influences of larval diet choice on biology of the butterfly and the risk of predation or parasitism. Lepidoptera species in particular have been the subject of tritrophic investigations regarding sequestration of host plant biochemicals for use as defensive substances.[14]

Chilades pandava male fitness may also be under the influence of larval food quality. We quantified the life span response to larval food, but the ability to sire offspring was not quantified. Because larval food quality affected fitness of the females, a similar response of the males may be shown with further research. Moreover, *C pandava* male longevity has been reported to exceed that of female longevity in Taiwan,[15] which differs from our results (Table 1). More case studies with diverse *C pandava* populations and more *Cycas* species may aid in understanding these contrasting experimental outcomes. For example, the containers used to determine longevity and fitness of the pair of Taiwan adults were only 11.5 cm in diameter.[15] The aerial courtship rituals of this butterfly prior to mating can be elaborate, and this ritual would not have been enabled by the small containers. The stress of being confined to such a small space may have been more detrimental to female health than male health.

Finally, as a group, cycads are highly threatened globally, and the relationships between each species and the other native organisms that form in situ interdependencies deserve to be studied before those relationships begin to falter due to anthropogenic threats. Numerous other Lepidopteran species rely on cycads as their exclusive host plant,[7,16] and more research on all of these species may improve our understanding of cycad biology and ecology.

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Author Contributions

TEM, AJL, and PNM conceived and designed the experiments; contributed to the writing of the manuscript; agree with manuscript results and conclusions; and made critical revisions and approved final version. TEM analyzed the data and wrote the first draft of the manuscript. TEM and AJL jointly developed the structure and arguments for the paper. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, authors have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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