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IS CYCASIN IN *Eumaeus minyas* (LEPIDOPTERA: LYCAENIDAE) A PREDATOR DETERRENT?

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... arvae exhibit a wide range of defensive strategies to avoid being eaten, e.g., mimetic coloration, shelter construction, unpalatability due to urticating hairs, spines and defensive glands, regurgitation, chemicals sequestered from host plants, secretion of volatiles, and noise production (see Brower, 1984; Bowers, 1993). Not only do they use their bad taste or unpleasant odor as a defense, they also announce it to potential predators by means of a conspicuous coloration, and gregarious and sedentary behavior (Bowers, 1993). Sequestration of defense compounds from larval host plants may require particular physiological adaptations by larvae to ingest, accumulate, and store those compounds (Brattsten, 1986; Bowers, 1992). Defense compounds are used for various purposes, particularly against predators, (Bowers, 1990; Duffey, 1980; Blum, 1983; Brower, 1984). Research on acquisition of chemical defenses by insects, particularly in Lepidoptera, has been done using adult individuals. However, it is usually during the larval stage that chemical defenses, sequestered from host plants, are ingested, processed and stored (Bowers, 1993). Examples of chemical defense of Lepidoptera larvae are well known (Brower, 1984; Bowers, 1990; Witz, 1990).

In aposematic species unpalatability is coupled with a warning coloration which can have many consequences for the biology and ecology of these species. Many “chemically defended” Lepidoptera are aposematic, and store plant compounds that are known vertebrate toxins, such as cardenolides (Brower, 1984), alkaloids (Rothschild et al., 1979; Boppre, 1990; Montllor et al., 1990), and cyanogens (Jones et al., 1962). Chemical defense of aposematic insects has also been shown to be effective against invertebrate predators, which can learn to subsequently avoid similar prey (Montllor and Bernays, 1993).

Cycasin is a secondary metabolite present in cycads (Cycadales), belonging to the azaoyglycosid group (Matsumoto and Strong, 1963; Whiting, 1963; Kobayashi and Matsumoto, 1965; Morgan and Hoffman, 1983; Norstog and Nicholls, 1997; Jones, 2000). Species of the American genera *Zamia*, *Ceratozamia* and * Dioon* (Cycadales: Zamiaceae) are hosts to aposematic butterflies of the genus *Eumaeus* (Lepidoptera: Lycaenidae). It has been demonstrated that *E. atala* sequesters cycasin from *Z. floridana*, which is later used as a defense against both vertebrate (Bowers and Farley, 1990) and invertebrate (Rothschild et al., 1986; Bowers and Larin, 1989) predators. These results were based on laboratory experiments, but no field research has confirmed it. It has also been suggested that cycasin works in *E. minyas* as a defense mechanism, providing chemical protection (Clark and Clark, 1991; Nash et al., 1992; DeVries, 1994). However, this has not been tested and the life cycle of *E. minyas* remains undescribed. We present the results of laboratory and field experiments to evaluate the protective function of cycasin in the aposematic butterfly *E. minyas*. In particular, the following questions are addressed: In which stages of its life cycle does *E. minyas* contain cycasin? Does pure cycasin repel potential predators of *E. minyas*? Does cycasin turn eggs, larvae and adults unpalatable? Is cycasin efficient in protecting eggs and larvae from their natural predators under natural conditions? This research is part of a wider study on the interactions between *E. minyas* and *E. debora* and their host plants *Z. lodgisii* and *D. edule*.

Materials and Methods

**Study site**

Field work was accomplished in an oak forest near Chavarrillo, in central Veracruz, México (19º24'N, 96º48'W; 1000m altitude), characterized by a calcareous-derived soil and abundant rock outcrops. The climate is temperate-humid, mean annual temperature is 24.5ºC, total annual precipitation is ca. 1110mm, with a rainy season between June and September and an extended six-month dry season (García, 1964; Soto et al., 1996). The veg-
ation is characterized (Flores, 1995) by a mixture of oak forest [Quercus oleoides, Q. laurina, Q. peduncularis (Fagaceae), Nectandra sanguinea (Lauraceae), Bursera simaruba (Burseraceae)] and palm groves of Brahea dulcis (Areaceae). Epiphytes are present in the Aracaeae, Bromeliaceae, Orchidaceae and Cactaceae (Castillo, 1985). The main shrubs and herbs are (Flores, 1995) Acacia cornigera, A. pematula (Mimosaceae), Zamia loddigesi, and Dioon edule (Zamiaceae).

Species

Zamia loddigesi (Zamiaceae, Miquel 1843) (voucher, L. M. White-lock 11/26/1963, XAL) is a small plant (up to 1m tall) with 1 to 6 fronds, inhabiting tropical dry and deciduous forests and secondary vegetation (Vovides et al., 1983). It is distributed along the coast of the Gulf of Mexico up to Guatemala, from 0 to 1000m in elevation (Vovides et al., 1983; Jones, 2000). Z. loddigesi is protected under the status of threatened species both internationally (CITES Appendix II) and nationally (Anonymous, 1994).

Eucaeus minyas (Lycce-nidae, Hübner 1809) is distributed from México to Costa Rica, where the larvae have been reported to consume fronds and female reproductive cones from Z. fur-furacea, Z. skinneri and Z. loddigesi (De-Vries, 1976; 1983a; Clark and Clark, 1991). E. minyas is considered aposematic, exhibiting a flashy warning coloration (De-Vries, 1977; Clark and Clark, 1991; Nash et al., 1992).

Chemical analyses

Chromatography on silica-gel plates (Whatman PE SIL G/UV) was used to determine presence or absence of cycasin in all stages of E. minyas. From individuals collected in November 1999, we used 36 eggs (0.0293g), 16 larvae of the four instars (2.045g), 1 pupa (0.1039g), and 5 adults (0.5351g). The fresh samples were processed using the technique described by Yagi et al. (1980) and Bowers and Larin (1989). For comparison we used standard pure cycasin (Biochemical 66950), and computed the reference factor (R) for each sample. Vouchers are deposited in a personal collection at the Departamento de Ecología Vege-tal, Instituto de Ecología, A.C.

Field experiments

Palatability experiments, modified from Bowers and Larin (1989) were conducted using individuals of Sole-nopsis geminata (Hymenoptera: Formicidae, Fabricius 1804) as predators. This ant inhabits the study site and was observed on Z. loddigesi. Before the experiments, ants were collected from a colony in Francisco Javier Clavijero Botanical Garden (Xalapa, Veracruz, México; 19º30’N, 96º57’W; 1280m altitude). The sample included workers, soldiers and larvae in order to ensure that ant behaviour was the least affected by sampling. (Jorge Valenzuela-González, personal communication). Ants were placed in 25 x 13cm plastic containers covered with mesh, and were deprived of food 48h before the experiments. The containers were then uncovered and placed within a square (0.26m²) of Tanglefoot® (Tanglefoot Co., Grand Rapids, MI, USA), where different food solutions were offered to the ants (see below).

To test if pure cycasin deterred S. geminata individuals, ants were offered two solutions: 1) control (0.75g sucrose + 0.30ml water), and 2) experimental (0.75g sucrose + 0.30ml of 1mg/ml cycasin in water). Cycasin was the same as that used for chemical analysis. The number of ant visits to each solution was recorded during 90 min. When an ant lowered its head and touched a solution, it was considered a visit. The experiment was replicated eight times with different ants in the same conditions mentioned above.

To test whether cycasin in eggs, larvae and adults rendered them unpalatable and deterred S. geminata individuals, individuals of E. minyas were collected in the study site: 33 eggs (0.02g), 15 larvae of the four instars (0.70g), and 2 adults (0.20g). Extracts were prepared with EtOH 70%, separately for each stage. These were used to prepare three experimental solutions (0.75g sucrose + 0.30ml of egg, larval or adult extract). Ants collected previously were offered four solutions, a control one (0.75g sucrose + 0.30ml water), and three experimental ones (with egg, larval or adult extract). This experiment was also replicated eight times, with different ant species in the same conditions and the number of ant visits to each solution was recorded during 90 min trial periods.

To test whether the presence of 70% ethanol used in the extraction procedure affected predator behavior, or if it masked the presence or cancelled out the effect of cycasin, we counted visits of individuals of S. geminata to a control (0.75g sucrose + 0.30ml water) and to an experimental solution (0.75g sucrose + 0.30ml 70% EtOH). The number of visits were transformed using the square root of X + 0.5, and no significant differences (ANOVA, F = 0.075, p = 0.7877) were found in the number of visits between solutions (control, X = 84.3755 ± 15.123, N = 675; experimental, X = 86.8755 ± 6.465, N = 695).

To test the defensive function of cycasin under natural conditions, a predator exclusion experiment was conducted at the study site. Egg clusters of E. minyas were located on individuals of Z. loddigesi and three predator exclusion treatments were applied: 1) fronds covered with mesh, 2) fronds with a band of Tanglefoot® at the base, and 3) fronds with Tanglefoot® and mesh; the control were fronds with egg clusters under natural conditions. Different treatments were used to determine differential effects of the different groups of predators (mainly for birds and ants, but other flying and crawling ins-ects as well). Eighty-three eggs (total for the study site at the time) grouped in egg clusters located on 15 Z. loddigesi individu-als were used. 32 on control plants and 19 with Tanglefoot® and mesh; 51 on treatment plants (16 with mesh, 19 with Tanglefoot®, 16 with Tanglefoot® and mesh). Over 15 days (the time for egg hatching), the plants were visited every day and the number of eggs preyed upon, hatched and un-hatched was registered. The exclusion experiment was continued applying the same treatments to the recently hatched larvae, plus 15 more first-instar lar-vae collected elsewhere in the study site. A total of 70 larvae, 26 on control plants and 44 on treatment plants (19 with mesh, 15 with Tanglefoot®, 10 with Tanglefoot® and mesh) were observed. The number of lar-vae that had disappeared, died or still sur-vived was counted daily. To establish the larval instar, larval length every third day until puation (ca. 16 days) was measured. Observations were extended up to 32 days because different-aged cohorts were ob-served. The number of resulting pupae and of larvae that reached adulthood was also counted.

Statistical analyses

The total number of visits to a particular solution was analyzed with a heterogeneity x²-test (Zar, 1999) to determine whether pure cycasin deterred potential predators. The number of visits to a particular solution were transformed (square root of X + 0.5) and then ana-lyzed with a one-way ANOVA (Abacus Concepts, Inc. 1996; Zar, 1999) to deter-mine whether the presence of cycasin in butterflies offered protection.

To test for the efficiency of cycasin as a chemical defense of eggs under natural conditions, the percentage of surviving eggs was computed. To test cycasin as a chemical defense of larvae in natural conditions, we used a ‘survival’
or ‘failure time’ analysis (Muenchow, 1986; Pyke and Thompson, 1986). The analysis calculates the probability of the occurrence of predation events during one observation period. The start of the observations was designated as time zero, and the predation events, disappearance of larvae, were subsequently counted over 16 days. If a given larva was preyed upon, it was considered an uncensored data point, and, if predation never occurred, i.e., the larvae survived, it was considered a censored data point. To compute the functions among variables the product-limit Kaplan-Meier nonparametric method (Abacus Concepts, Inc. 1996) was used, and the log-rank statistic (Mantel-Cox) was used to test for differences between the control and a given treatment (treatments were grouped). An among-treatments comparison showed that >20% of the events were censored and, as suggested by Pyke and Thompson (1986), instead of the survival analysis we used a 3x2 contingency table (Zar, 1999). All results are shown as mean ± standard error.

**Results**

**Life history**

The only herbivore observed foraging on the fronds of *Z. loddigesii* was *E. minyas*. During the rainy season *Z. loddigesii* is frequently visited by *E. minyas*. Similar to other caycads (Norstog and Nicholls, 1997), *Z. loddigesii* grows slowly and irregularly (only a few centimeters per year) and produces one to two fronds per year during the rainy season.

Adult butterflies of *E. minyas* oviposit on new fronds, of which larvae may consume up to 90% during their development. Larvae may also forage on the raquis and, while uncommon, butterflies may also oviposit on female reproductive cones, which are also harmed by larvae.

Eggs are deposited in small clusters (mean of egg by cluster= 5.238 ±0.547, N= 42), they are pale pink, turning white after one day, and hatch after approximately 11 days. Larvae are bright red with seven transversal yellow stripes and undergo four instar changes in 16 days. Pupae are orange with black marks, puation lasting approximately 19 days. Larvae and pupae are gregarious (DeVries, 1976; 1977; 1983b). The distinctive characteristics of adults are an orange-red abdomen, and black wings with metallic marks and white margins. It takes approximately 46 days from oviposition to adulthood.

**Cycasin detection and palatability experiments**

Cromatographic comparison indicated that cycasin was detected in all stages of the life cycle of *E. minyas*; standard Rf= 71.6; eggs= 71.0; larvae= 63.1; pupa= 53.7; and adults= 63.3. Variation in larvae and pupae was attributed to differences in the molecular structure of cycasin due to metabolic changes within a stage. There are some differences in pattern due to the metabolic conjugation in the insect of some of the plant components (Harborne, 1988). These variations in Rf were also observed in evaluations in the variations of cardenolide content of adult monarch butterflies by comparing the chromatographic separation of extracts from insects and from food plants (Harborne, 1988).

Visits of *S. geminata* were significantly higher to the water/sucrose solution than to the water/pure cycasin solution (x ²= 80.7249, p<0.001). Similarly, significant differences (ANOVA, F ⁰.⁰·² = 11.591, p<0.0001) were found (Figure 1) among ant visits to the different solutions prepared with *E. minyas* extracts and the control solution: sucrose/water (X ²= 132.37 ±19.77, N= 1059), egg extract (X ²= 43.50 ±4.89, N= 348), larva extract (X ²= 45.25 ±14.57, N= 362), and adult extract (X ²= 38 ±8.79, N= 304). There were significantly more visits to the water/sucrose solution (Fisher’s, p<0.001), and there were no significant differences among the other treatments (Fisher’s, p>0.05).

**In situ predator exclusion experiment**

No predation was found in the exclusions, as all of the 51 eggs in exclusion survived. However, out of 32 eggs in control plants 22 (68.75%) survived and 10 (31.25%) were predated. Three eggs fell from the frond due to heavy rain, 15 did not hatch, and 55 hatched (i.e., 66% passed from egg to 1° instar larvae). Hatching time was 11 days (X= 11.28 ±0.39, N= 18), and it took from one to four days for all eggs in a cluster to hatch (X= 1.85 ±0.27, N= 13).

Fifty six of the initial 70 larvae were killed. In 26 larvae of control plants, 25 (96.15%) were predated, and in 44 larvae of exclusion plants, 31 (70.45%) were predated. The number of surviving larvae (Figure 2) was significantly higher
and larvae were subject to attack by fungi like Beauveria bassiana (Bals.) Vuill., Deuteromycota. Egg mortality can also be attributed to factors intrinsic to the adult. For example, feeding quality and quantity while larvae or adult can significantly influence egg production and maturation (Scott, 1986; Brady and Jones, 1995). The egg stage is critical for the future survival of an E. minyas individual, the more eggs that hatch into larvae the higher the probability of surviving the larval stage, which, no doubt, is the most vulnerable and critical stage for the survival of an E. minyas individual (96.15% predation in the control).

The mortality rate of adults is important as every additional day of survival increases reproductive potential (Brower, 1984). Predation was significant on 3rd- and 4th-instar larvae (between 8 and 16 days of development). Under natural conditions, predation was significantly higher on non-protected larvae. However, predation in 1st- and 2nd-instar larvae did not differ between protected and non-protected conditions, and death of these larvae should be attributed to factors other than predation. Younger larvae are smaller and probably less conspicuous than older, larger, larvae (e.g., length of 3rd instar, X= 15.89mm ± 0.64, N= 42; 4th instar, X=21.97mm ± 0.64, N= 32; C. Castillo-Guevara, unpublished). No significant differences were obtained among exclusion treatments that would enable us to suggest the types of predators involved, although a number of ant species (Pheidole sp., Camponotus seri- ceiventris, Solenopsis geminata, and Ec tatoma tuberculatum, Hymenoptera: Formicidae), were collected in the study site. S. geminata and E. tuberculatum are carnivorous and potential predators (Valenzuela-González et al., 1995).

Each stage of the life cycle of lepidopterans has a specific guild of predators, and the mechanisms used to avoid them seem to be mostly related to visually-hunting vertebrate predators (Rausher, 1980; 1981). However, the first instars of many lepidopterans are predatory by a wide range of invertebrates, mostly night feeders (Dempster, 1984), and thus camouflage and warning coloration may not be a good defense mechanism.

Cannibalism was important to the survival of E. minyas larvae, especially when they were fully confined (Tanglefoot® + mesh treatment). Ingestion of conspecifics can result in a direct and crucial metabolic gain when lipid accumulation is not enough to support stress due to food shortage, but also in an indirect gain, the reduction of competitors (Polis, 1981; Wagner and Wise, 1996). Cannibalism in groups of E. minyas was also observed in Costa Rica and Panama; those slowest to molt were eaten by the fast-molting members (Phil DeVries, personal communication). We suggest that cannibalism among E. minyas larvae is a direct, easier and less energy-costly way to sequester cycasin.

As toxin levels vary considerably over short distances and through time, palatability to predators can also vary spatiotemporally (Harborne, 1999). Even though predators have developed behaviors and physiological responses to recognize unpalatable prey and thus decrease the effect of defenses (Calvert et al., 1979; Fink and Brower, 1981), a decrease in availability of palatable prey may induce predators to forage on less palatable prey (Alcock, 1970; Boyden, 1976). Moreover, young predator individuals may sporadically forage on unpalatable prey, or predators may forget a learned behavior (González et al., 1967).

It is not clear how cycasin works protecting lepidopterans against predators. The responses in predators include those caused by cardiac glycosides (vomiting) or pyrrolizidine alkaloids (bitter taste), which mask the real taste of cycasin (Nash et al., 1992). In addition, the presence of pyrazines (in E. atala) can enhance the protection offered by cycasin (Rothschild, 1984; Rothschild et al., 1986). Certain insects (e.g., Seiractia echo, Lepidoptera: Arctiidae) can circumvent the protective action of the cycasin produced by Zamia floridana (Teas, 1967), supporting the idea that no chemical defense against predators is absolute (Steward and Keeler, 1988; Agrawal, 1998). However, in the Eumaeus-Zamia interaction the level of cycasin is higher in Eumaeus than in the host plant (Rothschild et al., 1986). Production of secondary metabolites is not related only to defense against vertebrate or invertebrate herbivores, the system is even more complex and evidence for these substances under natural conditions is currently lacking and difficult to obtain. Furthermore, even though not tested, there seems to be a close association between warning coloration and unpalatability in E. minyas. First instar larvae are pale orange, and after three days of feeding on Z. loddigesi fronds and sequestering cycasin their color changes to bright red; consequently, they are more conspicuous.

Specialization on the Zamiaaceae and the warning attributes in Eumaeus larvae may suggest a long association through evolutionary time. However, protection by cycasin in eggs and
larvae could have evolved associated to predators that are currently extinct and thus extant predators are not so easily warned. Finally, we suggest that cyscin in the aposematic butterfly E. minyas has a defensive function against predators, however, its populations may be regulated by the negative effect of its predators, which apparently avoid the chemical defensive barrier.

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