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Two Genera of Aulacoscelinae Beetles Reflexively Bleed Azoxyglycosides Found in Their Host Cycads

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Abstract Aulacoscelinae beetles have an ancient relationship with cycads (Cycadophyta: Zamiaceae), which contain highly toxic azoxyglycoside (AZG) compounds. How these "primitive" leaf beetles deal with such host-derived compounds remains largely unknown. Collections were made of adult *Aulacoscelis appendiculata* from *Zamia* cf. *elegantissima* in Panama, *A. vogti* from *Dioon edule* in Mexico, and *Janbechynea paradoxa* from *Zamia boliviana* in Bolivia. Total AZG levels were quantified in both cycad leaves and adult beetles by high performance liquid chromatography (HPLC). On average, cycad leaves contained between 0.5–0.8% AZG (frozen weight, FW), while adult beetles feeding on the same leaves contained even higher levels of the compounds (average 0.9–1.5% FW). High AZG levels were isolated from reflex bleeding secreted at the leg joints when beetles were disturbed. Nuclear magnetic resonance and mass spectroscopy identified two AZGs, cycasin and macrozamin, in the reflex bleeding; this is the first account of potentially plant-derived compounds in secretions of the Aulacoscelinae. These data as well as the basal phylogenetic position of the Aulacoscelinae suggest that sequestration of plant secondary metabolites appeared early in leaf beetle evolution.

Key Words Aulacoscelinae · *Aulacoscelis* · Azoxyglycosides · Chrysomelidae · Coleoptera · *Dioon* · *Janbechynea* · Reflex bleeding · *Zamia* · Zamiaceae

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Introduction

One of the least understood plant-insect interactions is that between cycads and aposematic herbivorous beetles in the subfamily Aulacoscelinae (Chrysomelidae *sensu lato*), a basal lineage of leaf beetles closely associated with the neotropical cycad family Zamiaceae (Cycadophyta: Gymnosperm) (Windsor et al., 1999; Santiago-Blay, 2004). Cycads contain toxic azoxyglycosides (AZGs); herbivore defense compounds that have the common aglycone, methylazoxymethanol (MAM) (Bowers and Larin, 1989). The two most common cycad AZGs, cycasin and macrozamin (the β-glucoside and β-primeveroside of MAM, respectively), are found in the seeds and leaves of all cycad genera (De Luca et al., 1980; Yagi, 2004). These glycosylated storage forms are non-toxic but upon herbivory, endogenous plant or gut-associated enzymes cleave AZGs to the aglycone, which readily reacts with nucleic acids resulting in potentially mutagenic changes (Kobayashi and Matsumoto, 1965; Laqueur and Spatz, 1968; Schneider et al., 2002). Sequestration of AZGs from the host plant has been observed in a number of cycad-specialist herbivores. The larvae of the hairstreak butterfly (*Eumaeus atala florida* Poey, Lycaenidae: Lepidoptera) and of the

arctiid moth (*Seirarctia echo* Abbot and Smith, Arctiidae: Lepidoptera) feed on the leaves of *Zamia floridana* A.DC. In both herbivore species, the sequestered cycasin is retained in the adult stage where it is concentrated in the wings and abdomen (Teas, 1967; Rothschild et al., 1986). High β-glycosidase activity in the gut of the arctiid moth suggests that free MAM is re-glycosylated to restore cycasin after sequestration. Cycasin in *E. atala* deters ant predators and, probably, vertebrate predators as well (Bowers and Larin, 1989). The pollinating weevil of *Zamia furfuraceae* L. fil., *Rhopalotria mollis* Sharp (Belidae: Coleoptera), also sequesters cycasin from the male cones (Schneider et al., 2002).

Reflex bleeding refers to hemolymph-related secretions that are often associated with defensive posturing in many insect species. Aulacosceline adults exhibit a reflex bleeding mechanism similar to that observed in the Meloideae (Coleoptera) where upon disturbance of the insect, a droplet of hemolymph is exposed through the joints of the tibia and femur (Carrel and Eisner, 1974). The droplet is reabsorbed when the disturbance ceases. The purpose of this study was to determine whether AZG compounds are sequestered and secreted by cycad-feeding Aulacoscelinae leaf beetles.

Methods and Materials

Natural History Observations and Sample Collection Adult aulacosceline leaf beetles were collected from their respective cycad host plants by using a sweep net at three study sites: Huasteca Potosina, Mexico (May–September), Chagres National Park, Panama (May), and Potrerillos del Guenda, Bolivia (November) between 2008 to 2010. *Janbechynea paradoxa* Monrós and *Aulacoscelis appendiculata* Cox & Windsor suddenly appeared in large numbers as voracious feeders on the new leaves of *Zamia boliviana* (Brongn.) A.DC. in Bolivia and on *Zamia* cf. *elegantissima* Schutzman, Vovides and Adams in Panama, respectively. *Aulacoscelis vogtii* Monrós was observed in smaller numbers on *Dioon edule* Lindl. leaves during the rainy season in Mexico. Undamaged fully grown leaflets were collected and immediately frozen at −20°C until analysis. Adult beetles were removed from their host plants and the head, thorax, abdomen, and legs were manually separated using forceps. Three to four individuals of each species were pooled and frozen at −20°C for AZG analysis. Reflex bleeding was collected from live adults by gently holding the beetles with tweezers and transferring the droplets collected from the femur-tibia joints to filter paper by using capillary tubes. Beetle hemolymph was collected by centrifuging decapitated insects. The hemolymph and filter paper containing reflex bleeding were frozen at −20°C until extraction.

Azoxyglycoside Extraction and High Performance Liquid Chromatography Analysis Insect and leaf tissues were weighed and ground in 1 ml of chilled 70% ethanol. Homogenates were centrifuged at 13,000 rpm for 5 min, and the supernatant was split into subsamples; the first was used to measure basal levels of MAM and the second allowed measurement of AZGs through MAM equivalents. To measure AZG equivalents, the second subsample was diluted in sodium acetate buffer (pH 5.0) containing 0.27 U (enzyme unit) of β−glucosidase (Sigma) and incubated overnight in the dark at room temperature. Samples were syringe-filtered for HPLC analysis. Separation was achieved on a Cyano column (Varian Microsorb MV 100–5 CN, 150×4.6 mm) using a gradient from 75% methanol to 25% methanol in 10 min at a flow rate of 1.2 ml/min (Yagi, 2004). Peaks were monitored by a photodiode array detector at 215 nm. MAM standards were prepared by incubating commercially available MAM acetate (National Cancer Institute) in Tris–HCl buffer (final concentration 1 mg/ml, pH 8.0) with 36.9 U of esterase enzyme (Sigma) for 1 h at 25°C and used to generate a standard curve.

NMR and MS of Reflex Bleeding To identify the AZGs present in the reflex bleeding of *Janbechynea paradoxa*, four samples were pooled (approx. 10 μl), extracted in 50% methanol, and purified by HPLC. The concentrated sample was dried in a rotary evaporator and re-suspended in deuterated water for proton nuclear magnetic resonance analysis using a Bruker 300 MHz and accurate mass was obtained by sodium electrospray ionization ($[M+Na]^+$) MS/MS using an Agilent LS/MSD TOF mass spectrometer.

Results

Natural History Observations Little is known about the lifecycle of Aulacoscelinae beetles, except that the adults appear suddenly and for a brief period feed voraciously on the younger leaves of their cycad host plants during the onset of the rainy season (Windsor et al., 1999). Their long, sharp mandibles pierce the epidermis releasing plant juices that are then ingested. Mating occurs while the females are feeding.

Plant and Insect Azoxyglycoside Content The leaf AZG content of three Zamiaceae species ranged from 0.5–0.8% frozen weight (FW) on average (Table 1). *Dioon edule* showed the highest values (0.8%). For adult Aulacoscelinae beetles, the AZG content was higher, ranging from 0.9–1.5% FW. Trace amounts of AZGs were detected in the adult beetle head, thorax, abdomen, legs, and hemolymph.

Table 1 Azoxyglycoside content of adult Aulacoscelinae species and their respective host cycads

Insect Species	Fresh weight %±SD	*N*	Host Plant Species[b]	Fresh weight %±SD	*N*
Aulacoscelis appendiculata	1.49±0.77	4	*Zamia* cf. *elegantissima*	0.67±0.2	6
Aulacoscelis vogtii	1.12±1.04	4	*Dioon edule*	0.81±0.13	6
Janbechynea paradoxa	0.88±0.46	4	*Zamia boliviana*	0.52	2

[a] Whole body of adult insect. [b] Mature undamaged leaves

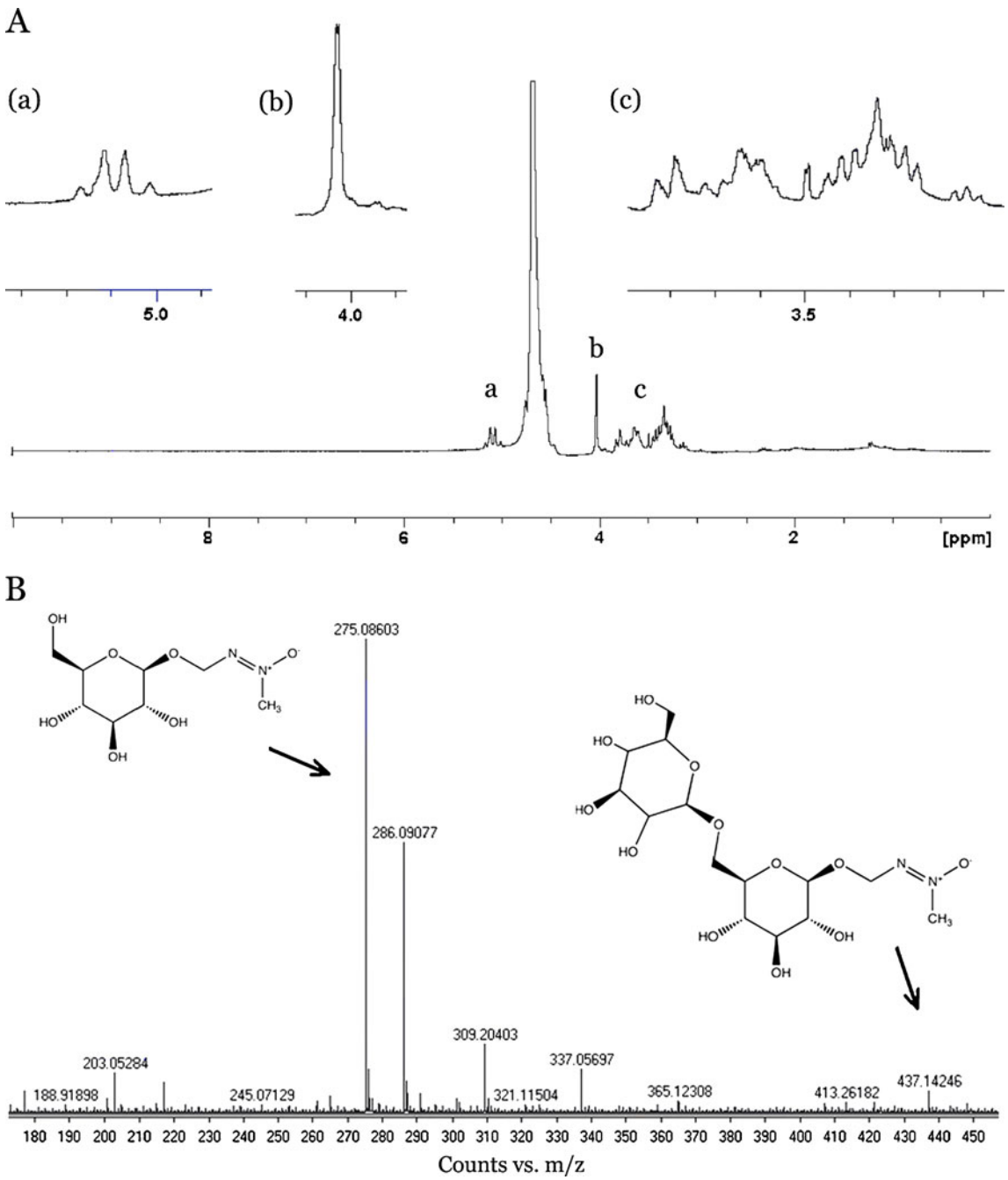

Fig. 1 Azoxyglycosides identified from the reflex bleeding of *Janbechynea paradoxa*. **A** ^{1}H-NMR spectrum of cycasin in deuterated water; *a*) anomeric CH, *b*) CH_3 and *c*) other CH, CH_2 and OH. **B** Accurate mass spectrometry of reflex bleeding; peaks corresponding to cycasin (275.096 m/z) and macrozamin (437.142 m/z) are identified

Azoxyglycosides in Adult Beetle Reflex Bleeding In response to disturbance, adult beetles secreted a droplet of reflex bleeding from the joint between the tibia and the femur. The color of the secretion ranged from clear to yellow and had a penetrating smell. The droplet was produced from any set of legs but usually was a localized response from the leg nearest the region of the body being held. Reflex bleeding (approx. 1.2 μl) was observed in all three Aulacoscelinae: *Aulacoscelis appendiculata, Aulacoscelis vogtii*, and *Janbechynea paradoxa.* Proton magnetic resonance spectra of secretions from *J. paradoxa* confirm that the reflex bleeding contained AZGs with diagnostic signals observed at 5.11, 4.03, and from 3.1 to 3.8 ppm (Fig. 1A). Mass spectroscopy identified a mixture of compounds with masses of 275.08, 286.09, 309.2, 337.06, and 437.14 *m/z*, indicating that the major component of the sample is cycasin (275.08603 *m/z*) with minor levels of macrozamin (437.14246 *m/z*) (Fig. 1B).

Discussion

Our observations suggest that toxic AZGs sequestered from the host plant may be part of the aulacosceline beetle defenses as they are exuded by the adult in response to disturbance (Fig. 1). Given the rarity of this class of compound in nature, it is likely that their abundance in insect herbivores reflects leaf chemistry as proposed by Schneider et al. (2002). Beetles were collected on specific plant species, and their sequestered AZG content is comparable to that of the host; however, in one of the field sites, Huasteca Potosina Mexico, another Zamiaceae species, *Ceratozamia mexicana,* grows near the *Dioon edule* population where insects were collected and might serve as an alternative AZG source for *Aulacoscelis vogtii.* The presence of both cycasin and macrozamin in the reflex bleeding suggests that these compounds are taken directly from the gut to the hemolymph through an active transport mechanism. A less likely possibility is that the sugar moiety is removed from AZG by glycosidases releasing toxic MAM that moves freely into the hemolymph, and then is re-glycosylated to form cycasin as proposed for *S. echo* caterpillars (Teas, 1967). Further research is being conducted to determine how Aucaloscelinae species accumulate these compounds and if they are capable of *in situ* synthesis.

As the strong smell of the secretion cannot be attributed to the AZGs, other plant- or insect-derived chemicals might also be part of the aulacosceline defense system. For example, AZGs are not the only compounds known to protect cycads against herbivores. The non-protein amino acid β-*N*-methylamino-L-alanine (BMAA) has the capacity to degenerate neural systems, and is found in several cycad tissues (Seawright et al., 1990). BMAA also could be part of the Aulacoscelinae defensive secretions, but this was not addressed in our study.

In an earlier review on defensive glands and secretions in the Chrysomelidae, Deroe and Pasteels (1982) lamented the lack of information available regarding Aulacoscelinae defense mechanisms. The authors proposed that for insects that do not have defensive glands associated with the surface of their elytra and pronotum as seen for other leaf beetle families, hemolymph-associated reflex bleeding may be an alternate defense mechanism. Here, we report that the Aulacoscelinae do not possess defensive glands in their elytra or pronotum but, in fact, reflexively bleed when disturbed. These secretions contain unusual AZG compounds also found in their host plants. The Aulacoscelinae is a sparse lineage of gymnosperm-associated herbivores, diverging alongside ancestors of the plant-feeding beetle superfamilies Chrysomeloidea and Cerambycoidea (Cox and Windsor, 1999). Putative Aulacoscelinae fossils date back to the Jurassic and earliest Cretaceous (Medvedev, 1968; Zhang, 2004). Therefore, the identification of plant-related secondary metabolites in Aulacoscelinae tissues and reflex bleeding opens the possibility that the ability to sequester plant-derived toxins may have appeared early in leaf beetle evolution, perhaps even predating the appearance of angiosperms.

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